THE CARDIOVASCULAR REGULATORY ROLE OF 5-HT IN THE NUCLEUS TRACTUS SOLITARIUS

A Thesis submitted for the Degree of
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to UCL

by

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I, Patrick Steven Hosford confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Patrick Steven Hosford
15th September 2013

ABSTRACT

The nucleus tractus solitarus (NTS) integrates afferent information to maintain cardiovascular homeostasis. Pharmacological experiments have indicated that 5-HT is released in this process. *In vitro* and *in vivo* experiments were carried out to measure this release. 5-HT was detected in the NTS indirectly, by measuring glutamatergic inward currents in the slice and directly by voltammetry in the anaesthetised rat.

In the slice, the presence of tonic release of 5-HT was confirmed which, in part, is responsible for basal release of glutamate via activation of 5-HT_3 receptors. Blockade of the high-affinity, low-capacity 5-HT transporter (5-HTT; SERT), with citalopram and the low-affinity, high-capacity transporters (organic cation transporter 3; OCT3 and the plasma membrane monoamine transporter; PMAT), with decynium-22 caused a 5-HT_{1A} mediated decrease in glutamate release. Evoked glutamate release was only augmented by decynium-22.

In experiments with anaesthetisia, a voltammetric scan optimised for 5-HT was used. Stimulation of cardiopulmonary, chemoreceptor and baroreceptor afferents as well as electrical stimulation of vagal afferents increased the electrochemical signal, which was calcium and frequency dependent. Synthesis inhibitors for 5-HT, but not for noradrenaline, decreased the signal confirming it was 5-HT. Decynium-22 increased the evoked signal but neither citalopram nor desipramine, at doses shown to selectively decrease the removal of 5-HT or noradrenaline, had any effect. Blockade of glutamate receptors with kynurenate reduced the evoked 5-HT by ~50% and this remaining 5-HT was potentiated by decynium-22.

Preliminary data from rats with heart failure induced by coronary artery ligation found that glutamate and 5-HT transmission to be augmented within the NTS.

These data show that 5-HT release is increased by activation of afferent input and this release is regulated by OCT3/PMAT not 5-HTT. This suggests that 5-HT may act, in part, as a volume transmitter in the NTS. This regulation may be affected by disease such as heart failure.

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ABBREVIATIONS

CNS	Central nervous system
COMT	Catechol *O*-methyltransferase
CVLM	Caudal ventrolateral medulla
DAT	Dopamine transporter
DVN	Dorsal vagal nucleus
eEPSC	evoked Excitatory postsynaptic current
EUS	External urethral sphincter
FCV	Fast-cyclic voltammetry
GABA	γ-aminobutyric acid
HDA	Hypothalamic defence area
HEK	Human Embryonic Kidney
HPLC	High-performance liquid chromatography
HR	Heart rate
i.a.	Intra atrial
i.p.	Intraperitoneal
i.v.	Intravenous
ID	Inner diameter
IPSP	Inhibitory postsynaptic potential
LSD test	Least significant difference test
MAO	Monoamine oxidase
mEPSC	miniature Excitatory postsynaptic current
min	Minutes
mRNA	Messenger ribonucleic acid
ms	milliseconds
NA	Nucleus ambiguus
NET	Noradrenaline or norepinephrine transporter
NTS	Nucleus tractus solitarius
OCT	Organic cation transporter
OD	Outer diameter
PKC	Protein kinase C
PMAT	Plasma membrane monoamine transporter

PNS	Peripheral nervous system
RT-PCR	Reverse transcription polymerase chain reaction
RVLM	Rosral ventrolateral medulla
s	Seconds
S.E.M.	Standard error of the mean
SCN	supra-chiasmatic nucleus
sEPSC	spontaneous Excitatory postsynaptic current
SERT	Serotonin (5-HT) transporter
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
VMAT	Vesicular monoamine transporter

ABBREVIATED COMPOUNDS

5-CT	5-Carboxamidotryptamine
5-HT	5-hydroytryptamine
5,7-DHT	5,7-dihydroxytryptamine
8-OH-DPAT	(±)-8-hydroxy-2-dipropylaminotetralin hydrobromide
ACh	Acetylcholine
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ATP	Adenosine-5'-triphosphate
BIMU 8	2,3-Dihydro-N-[(3-endo)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride
BRL-15572	3-(4-(4-chlorophenyl)piperazin-1-yl)-1,1-diphenyl-2-propanol
BW-723C86	5-((thiophen-2-yl)methoxy)-α-methyltryptamine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CP-809101	2-[(3-Chlorophenyl)methoxy]-6-(1-piperazinyl)pyrazine hydrochloride
DMSO	dimethyl sulfoxide
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DR4004	2a-(4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl)-2a,3,4,5-tetrahydrobenzo[cd]indol-2(1H)-one
EMD-386088	5-Chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole hydrochloride
EMDT	2-(2-ethyl-5-methoxy-1H-indol-3-yl)-N,N-dimethylethanamine
GBR-12909	1-[2-[Bis-(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride
GR-113808	1-methyl-1H-indole-3-carboxylic acid, [1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl ester
GR-127935	N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride
GR-55562	3-[3-(Dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride

GR-65630	(3-(5-methyl-1H-imidazol-4-yl)- 1-(1-methyl- 3H 3-1H-indol-3-yl)-l-propanone
L-694247	2-[5-[3-(4-Methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine
L-NAME	N (G)-nitro-L- arginine methyl ester
LP 12	4-(2-Diphenyl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride
LP 44	4-[2-(Methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1-piperazinehexanamide hydrochloride
LSD	Lysergic acid diethylamide
LY 344864	N-[(3R)-3-(Dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide hydrochloride
LY-334370	4-Fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]benzamide hydrochloride
mCPBG	1-(m-chlorophenyl)-biguanide
MDL-100907	([R(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol]
MDMA	methylenedioxymethamphetamine
MPP+	1-methyl-4-phenylpyridinium
PBG	1-Phenylbiguanide
PNU-109291	(S)-3,4-Dihydro-1-[2-[4-(4-methoxyphenyl)-1-piperazinyl]ethyl]-N-methyl-1H-2-benzopyran-6-carboxamide
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PTFE	Polytetrafluoroethylene
Ro 04-6790	4-Amino-N-[2,6-bis(methylamino)-4-pyrimidinyl]-benzenesulfonamide
RS-100235	1-(8-amino-7-chloro-1,4-benzodioxan-5-yl)-3-[[3-(3,4-dimethoxyphenyl)prop-1-yl]piperidin- 4-yl]propan-1-one
RS-102221	8-[5-(2,4-Dimethoxy-5-(4-trifluorom-ethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8--triazaspiro[4.5]decane-2,4-dione hydrochloride
RS-127445	4-(4-Fluoro-1-naphthalenyl)-6-(1-methylethyl)-2-pyrimidinamine hydrochloride

SB-204070	8-Amino-7-chloro-2,3-dihydro-1,4-benzodioxan-5-carboxylic acid, 1'-butyl-4'-piperidinylmethyl ester
SB-269970	(2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride
SB-271046	5-Chloro-N-[4-methoxy-3-(1-piperazinyl)phenyl]-3-methyl-benzo[b]thiophen-2-sulfonamide hydrochloride
SB-699551-A	3-cyclopentyl-N-[2-(dimethylamino)ethyl]-N-[(4′--4-biphenylyl)methyl]propanamide dihydrochloride
SR-57227	1-(6-Chloro-2-pyridinyl)-4-piperidinamine hydrochloride
ST-1936	5-Chloro-3-[2-(dimethylamino)ethyl]-2-methylindole oxylate
TEA	Tetraethylammonium
WAY-100635	*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexane carboxamide

PUBLICATIONS ARISING FROM THIS THESIS

Hosford P.S, Millar, J. & Ramage, A.G. (2011) The release of serotonin (5-HT) by vagal afferents in the nucleus tractus solitarius (NTS) detected by fast-cyclic voltammetry in anaesthetised rats. Program No. 283.11 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online

Hosford PS, Millar J, Ramage AG (2011) Characterization of vagal afferent-evoked 5 HT release detected by fast-cyclic voltammetry in the nucleus tractus solitarius (NTS) of the anaesthetised male rat. *Proc Physiol Soc* PC39.

Hosford PS, Millar J, Ramage AG (2012) Evidence that activation of both vagal and glossopharyngeal afferents cause the release of 5 HT in the NTS. *FASEB J* 26: 701.1.

Hosford PS, Ramage AG, Mifflin SW (2013) Evidence that 5-HT neurotransmission within the nucleus tractus solitarii (NTS) differentially modulates glutamate release via 5-HT_3 and 5-HT_{1A} receptors. *FASEB J* 27: 932.6.

Hosford PS, Mifflin SW, Ramage AG (2013). 5-hydroxytryptamine mediated neurotransmission modulates spontaneous and vagal evoked glutamate release nucleus tractus solitarii (NTS) *Journal of Pharmacology and Experimental Therapeutics*. To be submitted.

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1. GENERAL INTRODUCTION

1.1. 5-hydroxytrytamine

History, discovery and nomenclature

The first report, in Italian, of what became known as 5-hydroxytryptamine described a substance with vasoconstrictor properties isolated from enterochromaffin cells of the rabbit stomach (Vialli & Erspamer, 1933). The authors were unable to elucidate the full structure at that time. Later, in 1948 a vasoconstrictor substance was also isolated from serum and named *serotonin,* and in 1949 Rapport indicated that it could be 5-hydroxytryptamine (Rapport *et al.*, 1948, Rapport, 1949). Confirmation and synthesis of 5-hydroxytryptamine followed soon after (Hamlin & Fischer, 1951). *Enteramine* was also finally identified, attributing the pharmacological effects observed to one compound; 5-hydroxytryptamine (Erspamer & Asero, 1952). Herein the chemical name, 5-hydroxytryptamine (5-HT), will be used to refer to the compound described above. Soon after, 5-HT was found to be present in mammalian brain extracts (Twarog & Page, 1953) and was then isolated from the canine brain (Amin *et al.*, 1954). Finally, it was proposed that 5-HT and noradrenaline were two neurotransmitters with opposing actions in the CNS (Brodie & Shore, 1957).

1.2. 5-HT as a neurotransmitter

Synthesis

5-HT belongs to a family of neurotransmitter known as the monoamines. This family includes the catecholamines, derived from tyrosine; noradrenaline, dopamine and adrenaline. Histamine and trace amines (eg. octopamine) are additionally present in this group. 5-HT differs slightly in structure; as an indolamine it includes a five-membered ring containing nitrogen fused to a benzene ring. 5-HT is synthesised from typtophan. Biosynthesis is achieved *via* two enzymes; tryptophan hydroxylase and 5-

hydroxytryptophan decarboxylase. First, tryptophan is converted to 5-hydroxytryptophan and then the secondary product is converted to 5-HT. The conversion from tryptophan to 5-hydroxytryptophan is the rate-limiting step.

Storage

Once present in the cytoplasm vesicular monoamine transporter (VMAT) transports 5-HT against the concentration gradient into secretory vesicles under a proton electrochemical driving force (see Henry *et al.*, 1998, Blakely & Edwards, 2012). Presynaptic terminals store 5-HT in conventional clear vesicles and, less commonly, in large dense-cored vesicles (see Liu & Edwards, 1997). It has been estimated that each vesicle contains between 50-100 molecules of 5-HT. There is also evidence that dense core vesicles containing 5-HT also co-package peptides, such as substance P (Dey & Hoffpauir, 1986). Ascorbic acid has also been found to be present in the vesicles containing 5-HT. Ascorbic acid is a well-known antioxidant and may be acting as a preservative for the packaged 5-HT. It is unclear if ascorbate functions as a neuromodulator itself, but it has been proposed that ascorbate changes the redox potential of receptors (Rebec & Pierce, 1994).

The concept of vesicle 'pools' was initiated when an early study proposed that there are two distinct presynaptic stores of transmitter in the neuromuscular junction (Liley & North, 1953). It was found that repeated stimulation caused an initial rapid decay in response, which then quickly reached a steady state. This led to the concept of a 'readily releasable' fraction and a 'non-readily releasable' fraction of vesicles. Since then, with a combination of ultrastructural imaging and functional assays it is generally accepted that most synapses rely on three pools of vesicles (see Rizzoli & Betz, 2005, Alabi & Tsien, 2012). The readily releasable pool contains docked vesicles, which are immediately available for release but are rapidly depleted after brief stimulation (for example Stevens & Williams, 2007). They are most proximal to the presynaptic membrane and represent between 1 and 5% of the total vesicular load of any given terminal. The recycling pool maintains release under normal physiological conditions and accounts for 10-20% of the total number of vesicles. These vesicles replenish the readily releasable pool in a matter of seconds due to their high mobility in resting terminals. They are scattered more distal from the membrane

than the readily releasable pool. Lastly, the reserve pool accounts for the majority of the total vesicular load and is located most distal to the pre-synaptic membrane. These vesicles are released following intense simulation and often only when the recycling pool has been completely depleted (Richards *et al.*, 2003).

Release

Conventional action potential-dependent release of 5-HT from vesicles is, as for other transmitters, triggered by a rise in intracellular calcium concentration. This causes vesicle fusion with presynaptic membrane by conformational change of the SNARE complex leading to release of the vesicle content (see Ramakrishnan *et al.*, 2012).
Using fast-cyclic voltammetry and mathematical modelling it has been estimated that synaptic concentrations of 5-HT peaked at approximately 6mM in the dorsal raphé or substantia nigra (Bunin & Wightman, 1998). This concentration was found to drop to 20nM outside the synapse.

Action potential-independent release of 5-HT also occurs. Amphetamine and amphetamine derivatives such as MDMA cause release of 5-HT by this mechanism. These compounds are transported into the cytoplasm and by inhibition of the VMAT increase the intracellular concentration 5-HT. The increase in cytoplasmic 5-HT concentration causes the re-uptake transporters in the pre-synaptic membrane to operate in reverse and 5-HT is extruded from the cytoplasm as a result (see Blakely *et al.*, 2005). More recently, a role of action potential independent release of 5-HT has been shown in the dendrites of dorsal raphé neurons, which allows for increased spatial segregation of released transmitter (Colgan *et al.*, 2012).

Peripheral 5-HT

Peripherally, 5-HT is found in platelets and enterochromaffin cells. It is also found in gastrointestinal tract (in enterochromaffin cells), urinary system, kidneys, liver, heart and sympathetic nerves. It has been shown that 5-HT does not readily cross the blood brain barrier when administered peripherally but is metabolized before it can do so

(Lexchin *et al.*, 1977). Indeed, peripheral 5-HT is rapidly metabolized by monoamine oxidase, primarily in the endothelium of the lungs and liver, to 5-hydroxyindoleacetic acid (Verbeuren, 1989).

1.3. Central 5-HT

Anatomy

Following the suggestion that 5-HT is a neurotransmitter in the brain, the development of the Flack-Hillarp technique allowed central monoamine-containing cell groups to be visualised (Falck *et al.*, 1962). When exposed to formaldehyde vapour catecholamines (noradrenaline, dopamine) and 5-HT are converted to fluorescent compounds. This effect was used to demonstrate that 5-HT in the CNS is distributed in discrete cell groups, with the majority along the midline in a group known as the raphé nuclei (Dahlstrom & Fuxe, 1964). Later, immunohistochemical studies targeting 5-HT itself validated this distribution (Steinbusch *et al.*, 1978, Steinbusch, 1981). These 5-HT containing cells comprise of a small proportion of the total CNS neurons. Nevertheless, in the cortex of the adult rat there is an estimated 6 million per mm^3 5-HT positive-varicosities (Audet *et al.*, 1989).

Figure 1.1 shows the anatomical locations of the 5-HT-containing raphé nuclei in the rat. The dorsal raphé is the largest of these nuclei, and the most studied, but it is estimated that only 40-50% of neurons present contain 5-HT, as shown in the cat and rat (Wiklund *et al.*, 1981, Descarries *et al.*, 1982). Dopamine-containing neurons have also been identified around the midline of this nucleus (Ochi & Shimizu, 1978). Glutamate and GABA positive neurons are dispersed throughout the dorsal raphé, with the latter consisting of one population that is co-localised with 5-HT (Nanopoulos *et al.*, 1982, Belin *et al.*, 1983, Clements *et al.*, 1991). There is also evidence for neuropeptides; substance P is especially of interest as it is also co-localised with 5-HT (Hokfelt *et al.*, 1978). Situated just caudal and ventral to the dorsal raphé is the median raphé but only a minority of neurons contained within are 5-HT-positive (Wiklund *et al.*, 1981).

Contained in the more caudal aspect of the brain are the raphé magnus, pallidus and obscurus. Approximately 50% of cells within the raphé magnus contain 5-HT, 25% are positive for substance P and 25% are positive for thyrotropin-releasing hormone (TRH), with some of these cells found to express all three (Johansson *et al.*, 1981). The raphé magnus also contains a number of neuropeptides including encephalin and neurotensin (see Bowker *et al.*, 1988). The raphé obscurus continues caudally from the raphé magnus along the midline past the level of obex. The majority of the cells within this nucleus contain 5-HT and there is also evidence for the presence of the same neuropeptides found in the raphé magnus (Steinbusch & Nieuwenhuys, 1983). The raphé pallidus is found on the ventral surface of the medulla extending midway from the raphé magnus to the caudal extent of the raphé obscurus. Again, this nucleus contains both 5-HT-positive cells and neuropeptides (Steinbusch & Nieuwenhuys, 1983).

Connections

Projections from the dorsal and median raphé constitute the major ascending 5-HT innervation to rostral brain areas (Takagi *et al.*, 1980). These projections travel ipsilaterally within the median forebrain bundle parallel to noradrenaline-containing fibres. These two nuclei supply 5-HT innervation to the hippocampus and thalamus (Azmitia & Segal, 1978) as well as septum and midbrain (Swanson & Cowan, 1979, Steinbusch, 1981). On the other hand the caudate putamen (Jacobs *et al.*, 1978), substantia nigra (Wirtshafter *et al.*, 1987), hypothalamic medial preoptic area, suprachiasmatic nucleus and anterior area are thought to be supplied by the dorsal raphé alone (van de Kar & Lorens, 1979). Projections to the cortex from these nuclei were previously thought to be diffuse and lacking specific synaptic appositions (Descarries *et al.*, 1975). However, this is now known not to be the case as evidence of specific synaptic connections is well established (see Parnavelas & Papadopoulos, 1989).

The locus coeruleus receives innervation from dorsal, median and raphé magnus (Cedarbaum & Aghajanian, 1978, Morgane & Jacobs, 1979). Within the brainstem the facial (Aghajanian & McCall, 1980) and trigeminal nuclei (Schaffar *et al.*, 1984)

contain 5-HT positive terminals, however their origin is unknown since projections were not found from raphé areas (Travers & Norgren, 1983). The rostral ventrolateral medulla (RVLM), the site of the "vasomotor centre" (see later), also receives 5-HT innervation from the dorsal raphé (Underwood *et al.*, 1999). Sparse 5-HT innervation from the dorsal raphé is detected in the cerebellum (Takeuchi *et al.*, 1982).

Projections from the (caudal) raphé magnus, obscurus and pallidus are mostly descending. These nuclei supply 5-HT innervation to the substantia gelatinosa and intermediolateral nucleus of the spinal cord. Tract tracing experiments using horseradish peroxidase injection into these areas found labelling in all neurons of the caudal raphé, including those that are 5-HT positive (Bowker *et al.*, 1982). Additionally, anterograde transport studies showed connections from the raphé magnus and pallidus to the C1-3, A1 and A2 cell groups (Nicholas & Hancock, 1990).

5-HT positive terminals and fibres are reported in significant numbers throughout the nucleus tractus solitarius (NTS; site of termination of cardiovascular afferents, see later) in the rat and cat (Steinbusch, 1981, Maley & Elde, 1982). Ultra structural evidence shows the highest concentration of 5-HT terminals in the caudal regions of the medial NTS in the rat (Pickel *et al.*, 1984, Calza *et al.*, 1985, Curtis *et al.*, 2013). 5-HT containing neuronal connections between the NTS and raphé nuclei have been described by retrograde labelling. Dorsal raphé and raphé magnus, obscurus and pallidus all project to the NTS (Thor & Helke, 1987, Schaffar *et al.*, 1988). Primary afferent terminals originating from the nodose ganglia (the site of vagal afferent cell bodies) form the peripheral 5-HT input into the NTS (Nosjean *et al.*, 1990, Sykes *et al.*, 1994). The area postrema is known to contain 5-HT-positive cell bodies (Steinbusch, 1981). These cell bodies send 5-HT-containing projections to the NTS as well as the dorsal vagal nucleus (DVN) and nucleus ambiguus, the location of vagal parasympathetic preganglionic neurons (Shapiro & Miselis, 1985).

On the ventral surface of the brainstem the motor neurons in the ventral respiratory group receive 5-HT innervation from the raphé magnus, obscurus and pallidus of the rat (Holtman *et al.*, 1990). In the cat the nearby nucleus ambiguus has also been shown to be innervated by the medullary raphé (Holtman, 1988).

Functions

Central 5-HT-containing nuclei discussed above have been implicated in a wide range of physiological and behavioural functions including anxiety, depression, aggression, sleep, memory, and reward (see Lucki, 1998). These functions are beyond the scope of this thesis and will not be discussed further. However, many of these 5-HT-containing nuclei are involved in autonomic regulation and an overview of their roles will be discussed below.

Several studies have found that electrical and/or chemical stimulation of the dorsal raphé and median raphé produce predominantly pressor and sympathoexcitatory effects. Electrical stimulation of the dorsal or median raphé causes increases in blood pressure, but not heart rate, in anesthetised rats (Kuhn *et al.*, 1980). This effect was attenuated by depletion of 5-HT by pre-treatment with *para*-chlorophenylalanine (*p*-CPA) and prolonged by blockade of 5-HT re-uptake with fluoxetine, suggesting the effect is mediated by 5-HT. This agreed with the results of an earlier study (Smits *et al.*, 1978). Similar effects have been observed in cats by electrical and chemical stimulation of these nuclei (Piper & Goadsby, 1985). However, electrical stimulation also activates fibres of passage so experiments using this method should be interpreted with care, as many of these fibres cross the midline in the vicinity of these raphé nuclei. Chemical stimulation with glutamate or glutamate analogues does not attract this caveat. A more recent study stimulated the dorsal and median raphé by glutamate microinjection and found approximately 85% of injection sites evoked pressor responses, increased renal nerve activity and respiratory rate (Alvarenga *et al.*, 2005).

The raphé magnus, obscurus and pallidus were first reported to evoke sympathoinhibition when electrically stimulated, suggesting a role in autonomic regulation (Coote & Macleod, 1974). However, further study has failed to reach consensus on a defined role of these nuclei. Most studies report mixed cardiovascular and sympathetic responses. In anaesthetised rats stimulation of the caudal raphé produces sympathoexcitation (Zhou & Gilbey, 1995, Blessing & Nalivaiko, 2001) and sympathoinhibition (Coleman & Dampney, 1995). Studies investigating the effect of

these nuclei on cardiovascular reflexes found that stimulation of the nucleus raphé obscurus inhibited the bradycardia evoked by activation of the chemoreflex. (Weissheimer & Machado, 2007). Interestingly, this effect was found to be mediated by 5-HT_3 receptors located in the NTS, indicating that 5-HT is released here upon raphé obscurus activation. Equally, stimulation of the raphé pallidus caused inhibition of the cardiopulmonary reflex in anaesthetised rats (Edwards & Paton, 2000).

Figure 1.1 Central 5-HT containing cell groups

Schematic representation of central 5-HT containing cell groups (B1-9; after Dahlstrom & Fuxe, 1964) and their major projections in the rat brain. Not to scale.

5-HT containing cell groups are associated with the following nuclei according to the brain atlas of Paxinos and Watson (1998). The distribution of 5-HT-containing cells is sometimes diffuse and may be associated with more than one nucleus.

B1: Raphé pallidus nucleus
B2/4: Raphé obscurus nucleus
B3: Raphé magnus nucleus
B5/8: Median raphé nucleus
B6/7: Dorsal raphé nucleus
B8: Caudal linear nucleus
B9: Nucleus pontis oralis

MFB: Median forebrain bundle

3V: Third ventricle
4V: Fourth ventricle

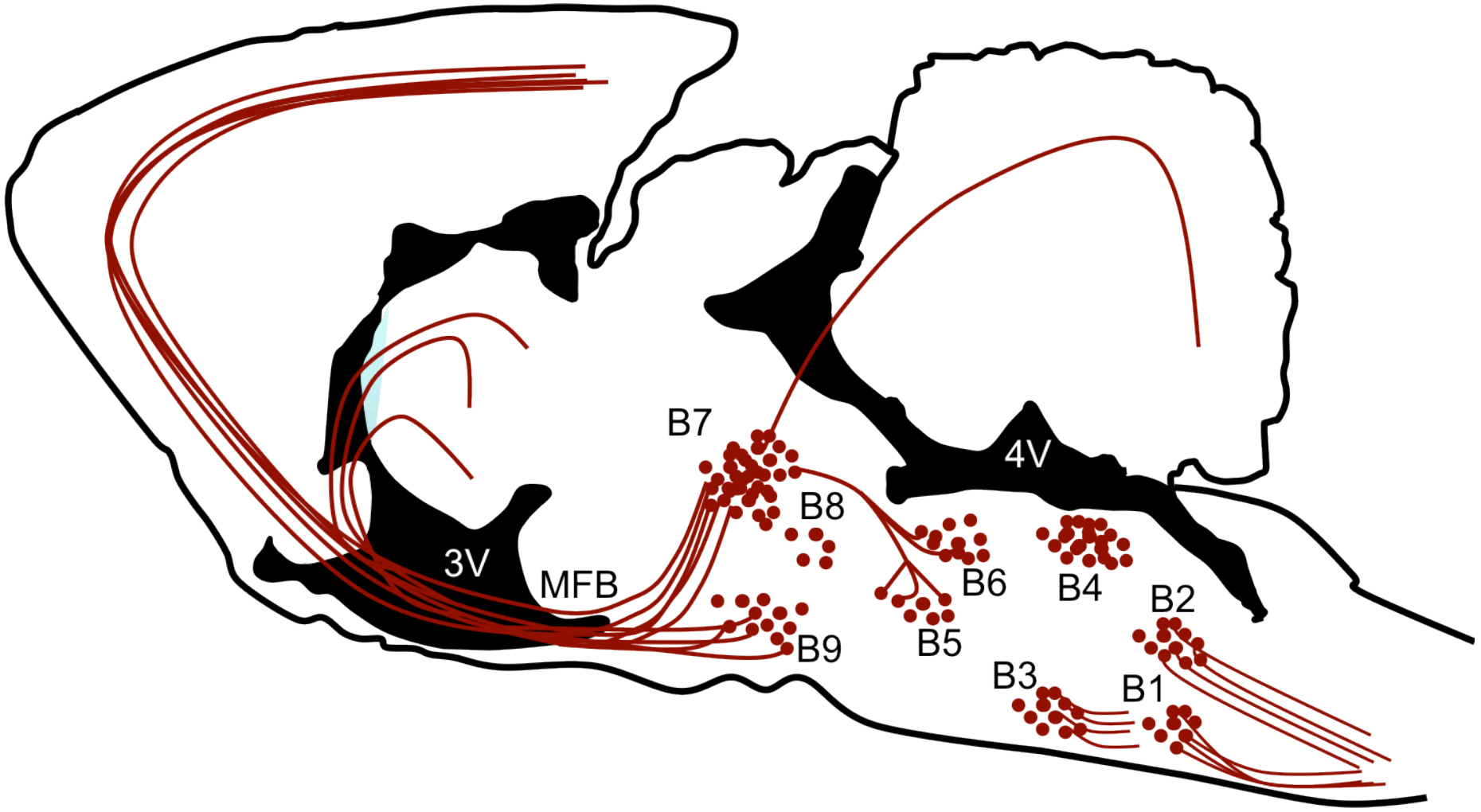
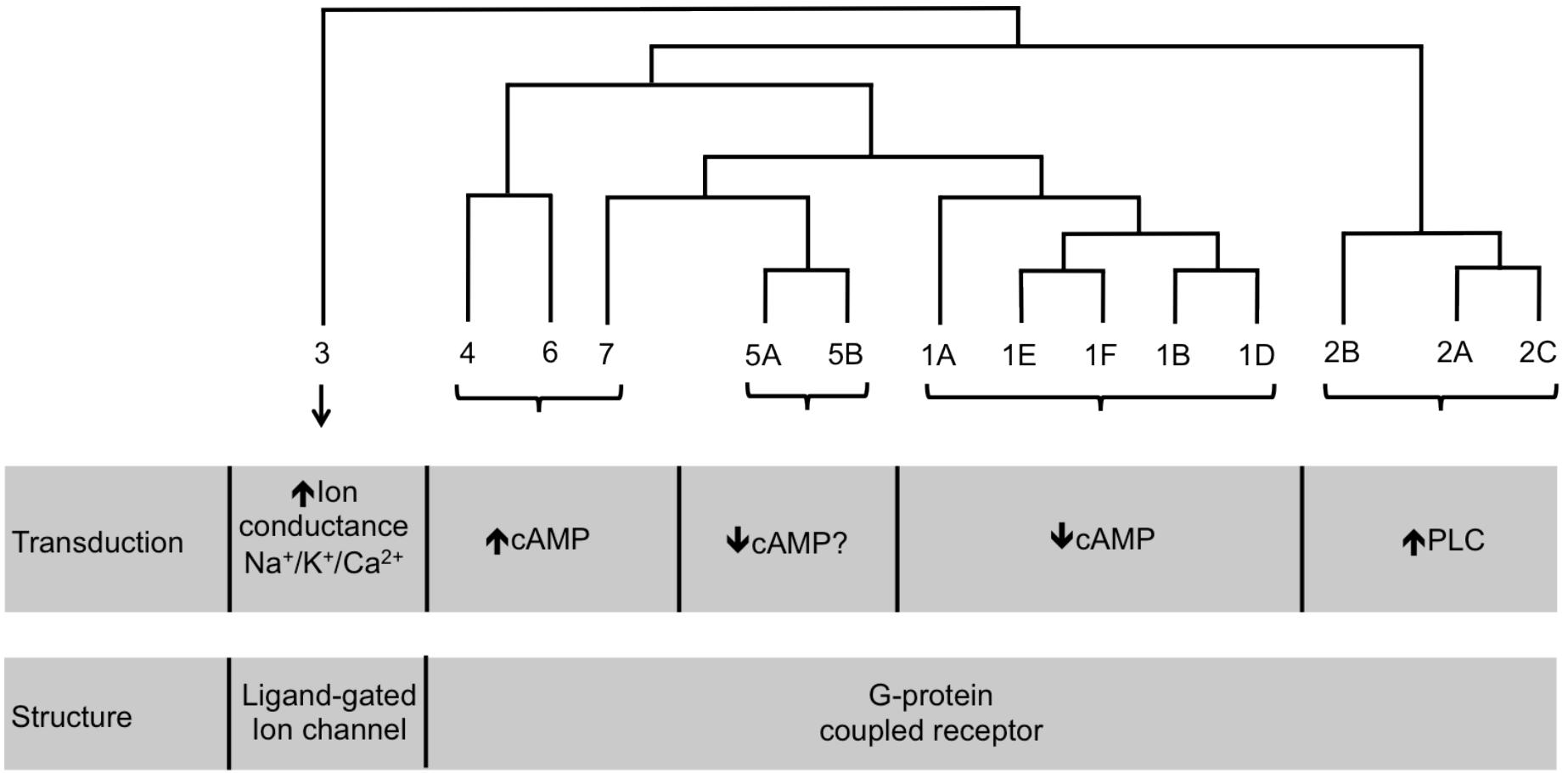

B7
B8
B9
B6
B5
B4
B2
B3
B1
3V
MFB
4V

Figure 1.2 5-HT receptors and their transduction systems

Dendrogram illustrating the evolutionary relationship between currently identified sub-classes of 5-HT receptor (adapted from Barnes & Sharp, 1999). Amino acid sequence homology compared in human genes, except 5-ht_{5A} and 5-ht_{5B} which are murine. The transduction mechanism and structure of each class of 5-HT receptor is also shown (Barnes & Neumaie, 2011).


3
4
6
7
5A
5B
1A
1E
1F
1B
1D
2B
2A
2C
Transduction
↑Ion conductance Na+/K+/Ca2+
↑cAMP
↓cAMP?
↓cAMP
↑PLC
Structure
Ligand-gated Ion channel
G-protein coupled receptor

1.4. 5-HT receptors

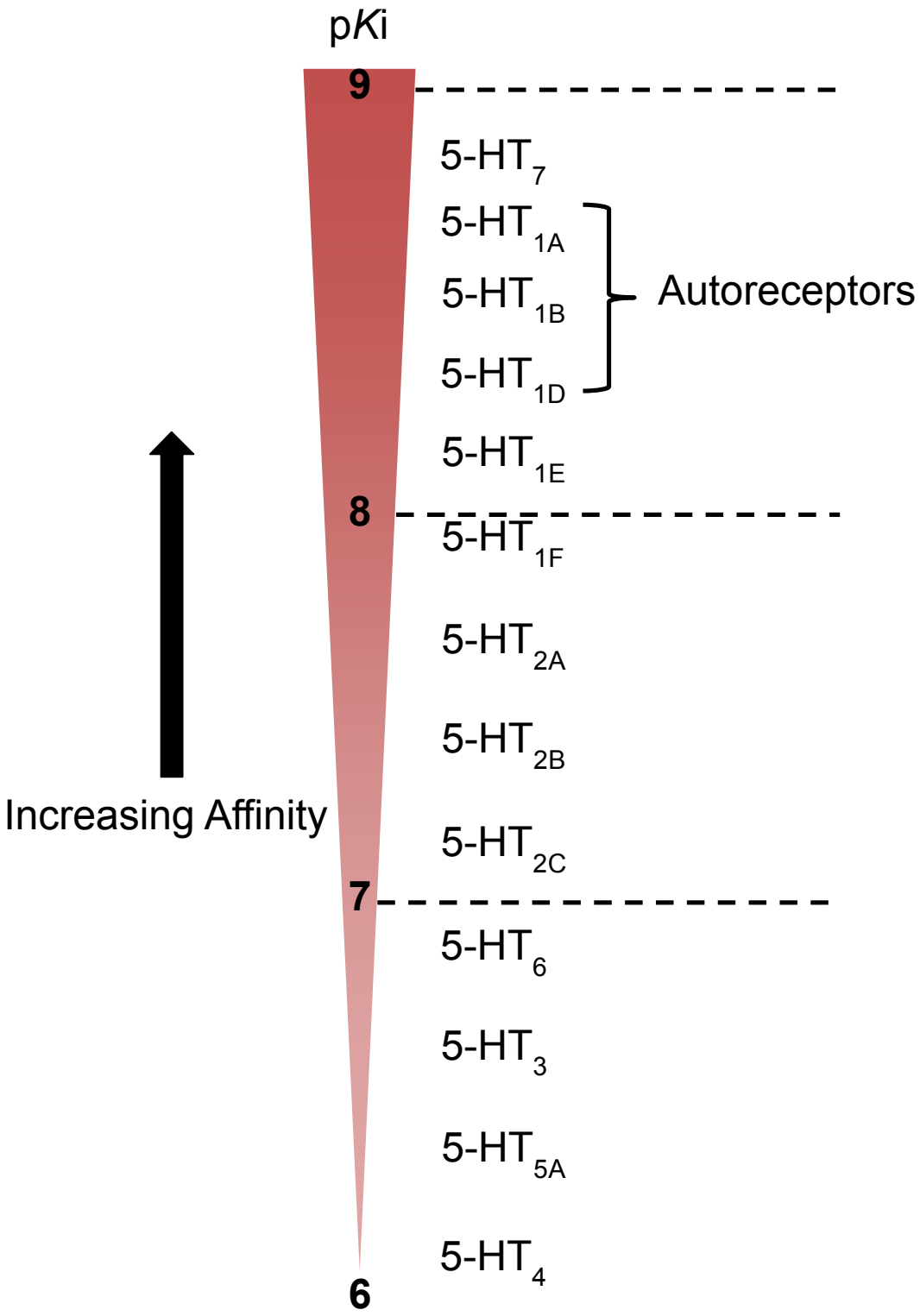
Study of the receptor pharmacology of 5-HT started when John Gaddam proposed that 5-HT acted on specific receptors; D (5-HT_{2A}) and M (5-HT_3) in the guinea pig ileum (Gaddum, 1953b). Further to this, lysergic acid diethylamide (LSD) was shown to antagonise the action of 5-HT on the rat uterus (Gaddum, 1953a). Now using dibenzyline (phenoxybenzamine; an α-adrenoceptor antagonist) and morphine to block the action of 5-HT on guinea pig ileum Gaddum and Picarelli (1957) proposed the existence of two classes of 5-HT receptor, D (5-HT_{2A}) on smooth muscle and M (5-HT_3) in the enteric nervous system. Since then 7 types of 5-HT receptor have been identified, some with different sub-types, splice variants and post-translational modifications (figure 1.2). This makes the 5-HT family of receptors one of the largest of all neurotransmitters (see Hoyer *et al.*, 1994) .

Affinity

5-HT receptors have differing affinities for their endogenous ligand. These differences allow further diversification of the role of 5-HT by allowing some receptors to respond at lower concentrations and others only at higher concentrations. Interestingly, receptors functioning as autoreceptors tend to display a higher affinity to 5-HT (see figure 1.3). Autoreceptors tend to be in extrasynaptic location and therefore experience lower concentrations of 5-HT.

Figure 1.3 5-HT binding to 5-HT receptors

Comparison of the affinity of 5-HT at cloned human receptors (data from Kilpatrick *et al.*, 1989, Roberts *et al.*, 2001). Functional, distributional and pharmacological evidence is available for 5-$HT_{1A/1B/1D}$ as autoreceptor. 5-HT_7 and 5-HT_{5A} receptors are possible autoreceptors due to pre-synaptic localization on 5-HT cells but no functional evidence is yet available.


pKi
9
5-HT7
5-HT1A
5-HT1B
5-HT1D
Autoreceptors
5-HT1E
8
5-HT1F
5-HT2A
5-HT2B
5-HT2C
7
5-HT6
5-HT3
5-HT5A
5-HT4
6
Increasing Affinity

1.4.1. 5-HT_{1A} receptor

Pedigo *et al.* (1981) first reported two populations of 5-HT binding sites in the cortex and striatum. [^{3}H]5-HT binding in these areas displayed very similar properties and were thought to be homogeneous. However, the authors noted a high affinity binding site in the cortex and a low affinity binding site in the striatum for the neuroleptic drug spiperone. These were later identified as 5-HT_{1A} and 5-$HT_{1B,}$ respectively. The 5-HT_{1A} receptor is one of the most extensively researched of all the 14 5-HT receptor subtypes due to availability of selective ligands and accessible experimental systems, such as the dorsal raphé electrophysiology.

Molecular biology

The 5-HT_{1A} receptor is a 7-transmembrane domain protein comprised of 422 amino acids. It was first identified as a result of human genome screens with a β_2 adrenoceptor clone, with which the 5-HT_{1A} gene shares similarities (Kobilka *et al.*, 1987). This intronless gene was found to be located on human chromosome 5 (q11.2 – 13) and was designated as the gene for the 5-HT_{1A} receptor (Fargin *et al.*, 1988). Soon after a gene was identified in the rat sharing 89% sequence homology that produced a 7-transmembrane domain protein again comprised of 422 amino acids that was identified as the rodent 5-HT_{1A} receptor (Albert *et al.*, 1990).

Activation of the cloned receptor expressed in HeLa cells found it to be negatively coupled to adenlyate cyclase (Pauwels *et al.*, 1993). Inhibition of cAMP accumulation by 5-HT_{1A} activation was found to be rapidly down-regulated after agonist exposure (van Huizen *et al.*, 1993). Additionally, there is evidence in hippocampal pyramidal cells that the 5-HT_{1A} receptor directly couples to potassium channels (Andrade *et al.*, 1986). The $G_{1\alpha}$ protein is also able to activate inward-rectifying potassium channels independent of cAMP inhibition (Codina *et al.*, 1987). Further, activation of 5-HT_{1A} receptors inhibit calcium influx (Penington & Kelly, 1990, Chen & Penington, 1996). Together it seems that direct coupling may account for a significant signalling method by the 5-HT_{1A} receptor.

Distribution

Autoradiographic studies have been used to form a comprehensive pattern of 5-HT_{1A} receptor expression within central structures. Using [^{3}H]-8-OH-DPAT the hippocampus was found to have the densest binding (Gozlan *et al.*, 1988). The same study also found binding in the lateral septal nucleus, cortex and hypothalamus in decreasing order of intensity. The dorsal raphé nucleus also displayed a high level of binding. The brainstem is also known to be rich in 5-HT_{1A} binding sites, with the DVN, NA and NTS having all been shown to express this receptor (Dashwood *et al.*, 1988). On the other hand, cerebellum and basal ganglia have few binding sites (Pazos & Palacios, 1985). Efforts to locate 5-HT_{1A} mRNA found a similar pattern as given by receptor binding studies. Wright *et al.* (1995) found mRNA expression in the following order of intensity; subiculum, entorhinal cortex, raphe, dorsomedial and ventromedial hypothalamus, with lower levels found in cortex, NTS, hypoglossal nucleus, spinal trigeminal nucleus, and the lowest in the cerebellum.

The subcellular location of the 5-HT_{1A} receptor is known to be postsynaptic in the hippocampus (Yocca *et al.*, 1992). However, in the dorsal raphé they function as somatodendritic autoreceptors, where their activation reduces neuronal firing (see Sharp *et al.*, 2007). Their role as autoreceptors is further supported by evidence that lesions of 5-HT-containing cells by 5,7-DHT abolish 5-HT_{1A} mRNA expression in the dorsal raphe (Miquel *et al.*, 1992).

Pharmacology

5-HT itself binds to the 5-HT_{1A} receptor with a pK_i of 8.8 at both rat and human receptors (Hoyer *et al.*, 1985, Hoyer *et al.*, 1986). There are several selective high affinity agonists available for the 5-HT_{1A}. The aminotetralins are the commonly used class of 5-HT_{1A} agonists. This includes 8-hydroxy-2-(di-n-proplylamino)tetralin (8-OH-DPAT; Arvidsson *et al.*, 1981), which has a pK_i of 8.5 at the rat receptor (Mellin *et al.*, 1991). However, 8-OH-DPAT has now also been shown to have agonist action at 5-HT_7 receptors (Lovenberg *et al.*, 1993). Busperione is an example of a 5-HT_{1A} partial agonist with a pK_i of 7.6 in rat cortex (Gozlan *et al.*, 1988). However, it is also

known to have affinity to the dopamine D_2 receptor and the α_1 adrenoceptor (see van Wijngaarden *et al.*, 1990).

Antagonist availability is also good. One of the most widely used antagonists is WAY-100635 (Forster *et al.*, 1995). This is a silent antagonist with a pK_i of 9.6 at rat cortical 5-HT_{1A} receptors (Johansson *et al.*, 1997). However, the same study noted affinity for the α_1 and D_2 receptors of pK_i 7.3 and 7.1, respectively. Other non-selective antagonists include the β-adrenoceptor antagonists (-)-pindolol and propranolol with pK_i's of 7.8 and 6.6, respectively (Hoyer *et al.*, 1985, Gozlan *et al.*, 1988).

General roles in physiology

Perhaps the best studied of all the 5-HT_{1A} receptor systems is that of the dorsal raphé. Here, 5-HT_{1A} is known to inhibit dorsal raphé neuronal firing in anaesthetised rats. This effect was first reported by Aghajanian *et al.* (1968) when systemic administration of lysergic acid diethylamide (LSD) was found to inhibit regular firing of 5-HT-containing neurons in the midbrain. This was later confirmed by iontophoretic application of the drug to these cells (Aghajanian *et al.*, 1972). Later, the more selective aggonist 8-OH-DPAT was used and produced a similar inhibition, which was blocked by (-)-propranolol and WAY-100635 (Sprouse & Aghajanian, 1986, Wang *et al.*, 1995). It seems that these 5-HT_{1A} receptors are tonically active as systemic (Gartside *et al.*, 1995) or iontophoretic (Wang *et al.*, 1995) application of WAY-100635 increases cell firing.

5-HT reuptake inhibitors have been found to reduce the activity of 5-HT-containing cells within the dorsal raphé (Gartside *et al.*, 1997). This effect is known to be mediated by the 5-HT_{1A} receptor as it is blocked by WAY-100635. This mechanism has been postulated to contribute to the clinical latency seen in the treatment of depression with these drugs (see Kinney *et al.*, 2000). 5-HT re-uptake inhibitors increase extracellular 5-HT concentration in the dorsal raphé leading to increased activation of 5-HT_{1A} receptors, reducing firing activity. Consequently, there is a reduction of extracellular 5-HT in the terminal fields in the forebrain. However, there

is evidence of 5-HT_{1A} receptor activation contributes to the antidepressant effect by increasing cellular proliferation in the dentate gyrus (Santarelli *et al.*, 2003).

5-HT_{1A} receptors modulate neurotransmitter release in a variety of systems. 5-HT efflux in the areas of the hippocampus (Sharp & Hjorth, 1990, Sharp *et al.*, 1996), frontal cortex, nucleus accumbens medial septum, and to a lesser extent the globus pallidus (Hjorth & Sharp, 1991) are all found to be reduced by 5-HT_{1A} activation. This is presumably due to activation of 5-HT autoreceptors in raphé nuclei (dorsal and median) that supply 5-HT innervation to these areas. Acetylcholine release is indirectly increased by 5-HT_{1A} activation in the rat frontal cortex (Consolo *et al.*, 1996b). The authours proposed that postsynaptic 5-HT_{1A} receptors are activated to increase dopamine release which, in turn, activate dopamine D_1 receptors to stimulate acetylcholine release. In the rat hippocampus noradrenaline release was enhanced by 8-OH-DPAT and this effect was block by administration of WAY-100635 (Hajos-Korcsok & Sharp, 1996). 5-HT_{1A} control of noradrenaline relase in the hippocampus was found to be insensitive to 5-HT depletion by p-CPA (Hajos-Korcsok *et al.*, 1999), suggesting it is not an indirect autoreceptor-mediated effect. Finally, there is some recent evidence that 5-HT_{1A} receptor activation inhibits glutamate release in the trigeminal neurons, at least in the slice preparation with GABA activity blocked (Choi *et al.*, 2013).

Role in autonomic control

The archetypical 5-HT_{1A} agonist 8-OH-DPAT has demonstrated the importance of these receptors in cardiovascular regulation. Activation of 5-HT_{1A} receptors at the level of the rostral ventrolateral medulla (RVLM; vasomotor centre) causes sympathoinhibition and a fall in blood pressure. However, blockade of these receptors with the 5-HT_{1A} antagonist WAY-100635 fails to cause an increase or change blood pressure indicating that these receptors are not under tonic activation (see Ramage & Villalon, 2008). These receptors are physiologically involved in reflex activation of parasympathetic outflow to cause vagal bradycardia by baroreceptor and cardiopulmonary reflex activation. The major site for this action is the nucleus ambiguus, which is also the site of origin of vagal preganglionic neurons (Wang &

Ramage, 2001). Although the NTS is also rich in 5-HT_{1A} receptors it is doubtful that these receptors are involved in afferent excitation of NTS neurons *in vivo* (Oskutyte *et al.*, 2009). The exact function of these NTS 5-HT_{1A} receptors in cardiovascular regulation remains to be determined. Another interesting possibility is that 5-HT_{1A} receptors could be involved in respiratory regulation. Indeed, 8-OH-DPAT microinjected into the dorsal vagal nucleus/NTS was found to increase phrenic nerve activity (Sporton *et al.*, 1991). In this respect 5-HT pathways are considered to play an important role in respiratory regulation (Hodges & Richerson, 2008).

1.4.2. 5-HT_{1B} and 5-HT_{1D} receptors

Before cloning was possible 5-HT_{1B} and 5-HT_{1D} receptors were originally thought to be one class of receptor but with differing interspecies pharmacology. Rodents were found to have high levels of 5-HT_{1B} binding whereas human, dog, calf and guinea pig displayed high 5-HT_{1D} binding (Hoyer & Middlemiss, 1989). Two human receptor genes were initially cloned and, when expressed, gave rise to receptors with a similar pharmacological profile as the 5-HT_{1B} in the rat (Hartig *et al.*, 1992). These were designated 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. However, when the rodent 5-HT_{1B} receptor was cloned it was found to have high sequence homology with the human 5-$HT_{1D\beta}$ (Adham *et al.*, 1992, Jin *et al.*, 1992). Closing the circle, a receptor with high sequence homology to the human 5-$HT_{1D\alpha}$ receptor was cloned in rat (Hamblin *et al.*, 1992) and nomenclature was adjusted accordingly. When cloning data was complete it became clear that there were two specific receptors subtypes, 5-HT_{1B} and 5-HT_{1D}, expressed by all species but with different distributions and differing pharmacology. For example the rat 5-HT_{1B} receptor has a higher affinity for beta-adrenoceptor antagonists such as isomoltane, pindolol and propranolol than the human receptor. This difference is conferred by a single amino acid change in the gene sequence (Oksenberg *et al.*, 1992).

5-HT$_{1B}$ receptor

5-HT$_{1B}$ receptor distribution has been assessed by autoradiography and immunohistochemistry. High levels of [^{125}I]CYP binding in the presence of isoprenaline were found in the rat basal ganglia, especially globus pallidus and substantia nigra (Pazos & Palacios, 1985). Lower densities were also found in entopeduncular nuclei, superficial gray layer of the superior colliculus and periaqueductal gray. 5-HT$_{1B}$ binding in the cerebral cortex, amygdala and hypothalamus was present but of lower density. Immunocytochemistry confirmed this distribution (Sari *et al.*, 1997, Sari *et al.*, 1999). There are some differences between the mRNA distribution and the receptor protein. *In situ* hybridisation studies failed to detect transcripts in the globus pallidus and substantia nigra where high levels of the receptor are expressed (Bruinvels *et al.*, 1994). This suggests that 5-HT$_{1B}$ is mainly located on nerve terminals in these areas as the protein is manufactured in the cell body and then transported to where it is functionally active. This was confirmed by studies looking at the subcellular location of the 5-HT$_{1B}$ receptor. One group showed exclusive expression on axons and axon terminals in the substantia nigra and globus pallidus (Sari *et al.*, 1997, Sari *et al.*, 1999). When taken together, the data supports a post-synaptic and pre-synaptic receptor expression functioning both as a heteroreceptor and autoreceptor.

Pharmacology of the 5-HT$_{1D}$ receptor is complex. Human 5-HT$_{1B/1D}$ receptors share a 77% sequence homology in their transmembrane domains so, unsurprisingly, ligand selectivity between the two sub-types is poor. The two receptors display binding profiles that are almost indistinguishable (see Weinshank *et al.*, 1992, Boess & Martin, 1994). Many ligands with affinity for the 5-HT$_{1B}$ also have affinity for the 5-HT$_{1B}$ or 5-HT$_{1A}$ receptors so combinations of ligands can be used effectively to distinguish between receptor sub-types. Examples of agonists available are sumatriptan, the archetypal 5-HT$_{1A/1B/1D}$ agonist and CP 94253, a potent and selective 5-HT$_{1B}$ agonist. Antagonists include SB-216641, which displays a 25-fold selectivity over human 5-HT$_{1D}$ receptors (Price *et al.*, 1997) and SB-236057, which has a 60-fold selectivity over other 5-HT receptors (Selkirk *et al.*, 1998).

5-HT_{1B} receptors are well known to act as inhibitory autoreceptors to modify 5-HT release in a range of brain areas. In this respect much of this research has been in an attempt to understand the clinical latency of 5-HT re-uptake inhibitors as a treatment for depression. When these uptake inhibitors are administered acutely, activation of pre-synaptic autoreceptors limits the increase in extracellular 5-HT concentration. The 5-HT_{1B} receptor has been shown to act as such in the forebrain and hippocampus of the mouse when treated with paroxetine (Malagie *et al.*, 2001). More recently, endogenously released 5-HT was shown to activate the 5-HT_{1B} receptor to reduce 5-HT release in the substantia nigra (Threlfell *et al.*, 2010). The study used voltammetry to detect electrically evoked 5-HT release in rat brain slices.

Various examples of the 5-HT_{1B} receptor acting as a heteroreceptor have been reported. Evidence modifying the release of acetylcholine, dopamine, GABA and glutamate. In synaptosomes prepared from the rat hippocampus, 5-HT_{1B} receptor activation reduced efflux of radiolabelled acetylcholine (Maura & Raiteri, 1986). Conversely, 5-HT_{1B} receptors are also known to have a facilitatory effect on the release of acetylcholine and dopamine in the rat frontal cortex (Consolo *et al.*, 1996a, Iyer & Bradberry, 1996). However, given that 5-HT_{1B} activation inhibits adenylyl cyclase this effect could be explained by disinhibition, possibly reducing GABA release. There is also direct evidence of 5-HT_{1B} modulating GABA release. One study found that 5-HT_{1B} receptors were responsible for the 5-HT-mediated inhibition of $GABA_B$ IPSPs in the rat midbrain (Johnson *et al.*, 1992). Finally, 5-HT_{1B} activation *in vitro* was found to inhibit glutamate release in the rat cingulate cortex and subicular cortex (Tanaka & North, 1993, Boeijinga & Boddeke, 1996).

5-HT_{1D} receptors

5-HT_{1D} receptors, like all in the 5-HT_1 class, negatively couple to adenylyl cyclase. Unlike 5-HT_{1B} receptors they are expressed at much lower levels in the CNS. Indicated by *in situ* hybridization 5-HT_{1D} receptors are distributed in the basal ganglia, hippocampus, cortex, dorsal raphé and locus coeruleus. However, low densities of 5-HT_{1D} mRNA is distributed throughout the CNS, including brainstem.

Sub-cellular distribution is thought to be predominantly on axon terminals of both 5-HT and non 5-HT-containing neurons.

Pharmacology of the 5-HT_{1D} receptor is complex. Human 5-$HT_{1B/1D}$ receptors share a 77% sequence homology in their transmembrane domains so ligand selectivity between the two sub-types is poor. The two receptors display binding profiles that are almost indistinguishable (Weinshank *et al.*, 1992, Boess & Martin, 1994). Many ligands with affinity for the 5-HT_{1D} also have affinity for the 5-HT_{1B} or 5-HT_{1A} receptors so combinations of ligands have to be used in order to effectively distinguish between receptor sub-types. Examples of agonists available are sumatriptan, the archetypal 5-$HT_{1A/1B/1D}$ agonist and PNU-109291, a potent and selective 5-HT_{1D} agonist with ~600 –fold selectivity over 5-$HT_{1A/2A}$ receptors (Ennis *et al.*, 1998). Antagonists include BRL-15572 which displays approximatley 60-fold selectivity for the 5-HT_{1D} receptor over the closely related 5-HT_{1B} subtype (Price *et al.*, 1997) and GR-127935 which is a 5-$HT_{1B/1D}$ antagonist with greater than 100-fold selectivity over other 5-HT subtypes (Clitherow *et al.*, 1994).

Supporting a role as an autoreceptor, one *in vitro* study on the found that 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} regulated the release of 5-HT. Using voltammetry to measure extracellular 5-HT concentration in the rat dorsal raphé nucleus they concluded that, when 5-HT reuptake was blocked with paroxetine, all three receptors were activated to inhibit release (Davidson & Stamford, 1995b, a). Further, the reduction of 5-HT release induced by the 5-$HT_{1B/1D}$ receptor agonist sumatriptan persisted in 5-HT_{1B} knockout mice (Pineyro *et al.*, 1995). There is also *in vivo* evidence supporting this observation; electrophysiological recordings from 5-HT-containing neurons were used to demonstrate the presence of the 5-HT_{1D} receptor (Pineyro *et al.*, 1996). However, this effect was not found under similar experimental conditions in a more recent study (Trillat *et al.*, 1997).

There is also evidence that 5-HT_{1D}, acting as a heteroreceptor, regulates the release of glutamate, GABA and acetylcholine. Initially, using synaptosomes prepared from rat cerebellar tissue one group showed a receptor with 5-HT_{1D} characteristics inhibit glutamate release (Maura & Raiteri, 1996). This was later followed up on tissue from the human cerebral cortex using the same method (Maura *et al.*, 1998). The same

research group also showed that glutamate release evoked by nitric oxide in rat cerebellar slices was regulated by a presynaptic 5-HT_{1D} receptor (Marcoli *et al.*, 2006). 5-HT_{1D} activation can also cause disinhibition by reducing GABA release. Once such example was reported in the human cortex where a 5-HT_{1D} -like receptor was found to exert an inhibitory action on GABA release *in vitro* (Feuerstein *et al.*, 1996). Further, both 5-HT_{1D} and 5-HT_{1B} were shown to inhibit GABA release in slices containing the periaqueductal grey area of the rat. The selective agonist 5-HT_{1D} L-694247 caused a decrease in GABA-mediated inward currents recorded in PAG neurons (Jeong *et al.*, 2008). Finally, synaptosomes prepared from the guinea pig hippocampus were found to have a 5-HT_{1D}-like receptor that, when activated, inhibited acetylcholine release (Harel-Dupas *et al.*, 1991).

Attributing these functional observations to behavioural or wider physiological effects is problematic due to these so-called selective ligands being relatively poorly characterized and the interspecies differences between the 5-HT_{1B} and 5-HT_{1D} receptors. The 5-HT_{1D} is often present with other 5-HT_1-type receptors making it difficult to discriminate specific functions. The reason for multiple sub-types controlling the same function remains to be determined and will require more selective ligands.

Role of 5-$HT_{1B/1D}$ in autonomic control

Expressed in peripheral cardiovascular tissues, 5-$HT_{1B/1D}$ receptors are known to modulate tone of a range of vessels either by direct actions on vascular smooth muscle or by a presynaptic sympathoinhibitory action (see Villalon & Centurion, 2007). In cats 5-HT inhibits transmission in the sympathetic ganglion which was attenuated by the 5-$HT_{1B/1D}$ antagonist GR-127935, but not other 5-HT receptor antagonists (Jones *et al.*, 1995). 5-HT_{1D} receptor activation has also been shown to inhibit noradrenaline release in the human atrium (Molderings *et al.*, 1996).

Centrally, within the NTS the iontophoretic application of the 5-$HT_{1B/1D}$ receptor agonist sumatriptan inhibited the firing rate of most neurons tested. When 5-HT_{1D} was

blocked with ketanserin, which has no effect alone, the inhibition was attenuated. In the presence of the 5-HT_{1B} antagonist GR-55562 the inhibition was potentiated (Jeggo *et al*., 2007). In the same study, sumatriptan decreased vagal afferent activity evoked by electrical and pharmacological (cardiopulmonary afferent) stimulation of the vagus nerve. Conversely, activation of the 5-HT_{1B} receptor was found to increase the same evoked activity. It was concluded that the two sub-classes had opposing actions in the NTS indicating different anatomical locations. In an earlier study central administration of sumatriptan attenuated the reflex response to smoke stimulation of the upper airway of the anaesthetised rabbit (Dando *et al*., 1998).

Overall, data indicate the presence of both 5-HT_{1B} and 5-HT_{1D} receptors in central and peripheral autonomic systems, possibly with opposing actions.

1.4.3. 5-ht_{1E} and 5-HT_{1F} receptors

5-ht_{1e} receptors

Lower case lettering of this receptor indicates that no physiological function have been identified. The gene for this receptor has been cloned from human genomic libraries (Levy *et al.*, 1992, Zgombick *et al.*, 1992) and the guinea pig (Bai *et al.*, 2004), but not detected in rat and mice. However, little is known about the physiology of the receptor due to lack of selective ligands. Most 5-HT_1 receptor ligands are known to have affinity at this receptor (Bai *et al.*, 2004).

5-HT_{1F} receptors

When the human gene was first detected in 1993 the 5-HT_{1F} became the 5th receptor in the 5-HT family to negatively couple adenylyl cyclase (Adham *et al.*, 1993).
Distribution of mRNA revealed 5-HT_{1F} transcripts at modest levels in hippocampus (CA1–CA3 cell layers), cortex (highest in the cingulate and entorhinal cortices) and dorsal raphé nucleus of the guinea pig (Bruinvels *et al.*, 1994). Autoradiography with

the selective agonist LY-334370 found a similar distribution in rat, guinea pig, monkey and human brain (Lucaites *et al.*, 2005).

The 5-HT_{1F} receptor is expressed on both 5-HT-containing and non-5-HT-containing cell bodies where it acts as both an autoreceptor and heteroreceptor, respectively. 5-$HT_{1B/D/F}$ receptors have been colocalised using immunohistochemical staining with glutamate-containing neurons (Ma, 2001). This suggests a role in controlling glutamate release, at least in the trigeminal nucleus.

Research interest in the 5-HT_{1F} receptor was fostered due to the anti-migraine effects of the triptan class of anti-migraine drugs. Triptans (sumatriptan, etc..) are non-selective 5-$HT_{1B/D/F}$ agonists and development of sub-type selective agonists to improve therapeutic profile of this target has lead to the development of high-affinity, selective 5-HT_{1F} agonists LY 344864 and LY 334370. The latter has ~100 fold selectivity of 5-HT_{1B} receptors and have been shown to be effective against migraine in animal models, however acting in the trigeminal nucleus and not at vascular sites, as previously thought (Phebus *et al.*, 1997). No selective antagonists have been developed to date and thus made research into the physiological role of these receptors difficult.

1.4.4. 5-HT_2 receptors

There are three identified sub-types of the 5-HT_2 receptor designated 5-$HT_{2A,}$ 5-HT_{2B} and 5-HT_{2C}. When activated all 5-HT_2 receptors increase the hydrolysis of phosphoinositide and induce a rise in intracellular Ca^{2+} concentration. Historically first reported as 5-HT 'D' receptor, which mediated the contraction of guinea pig ileum smooth muscle caused by 5-HT (Gaddum & Picarelli, 1957). This was later classified as the 5-HT_{2A} receptor. The 5-HT_{2B} receptor was originally described in the rat fundus and was given the designation 5-HT_{2F} until nomenclature was agreed. As previously mentioned, the 5-HT_{2C} receptor was originally known as the 5-HT_{1C} receptor but reclassified due to similar pharmacology and second messenger transduction.

5-HT_{2A} receptor

The receptor was first reported in rat cortical membranes to have a high affinity for spiperone but a relatively low affinity for 5-HT itself (Peroutka & Snyder, 1979). Approximately a decade later the receptor was cloned from rat and human cDNA libraries (Pritchett *et al.*, 1988, Julius *et al.*, 1990). Autoradiographic studies using [^{125}I]-DOI and [^{3}H]-MDL-100907 found high 5-HT_{2A} receptor binding in cortical areas, caudate nucleus, nucleus accumbens, hippocampus, and brain stem; particularly pontine, trigeminal motor and facial nuclei (Mengod *et al.*, 1990b, Lopez-Gimenez *et al.*, 1997). The distribution of the radioligand binding was well matched with mRNA distribution (Pompeiano *et al.*, 1994). Interestingly, one of the lowest levels of mRNA in the brain stem was found to be the NTS. Mostly overlapping mRNA and ligand binding sites indicate that the 5-HT_{2A} receptor is located post-synaptically to 5-HT-containing terminals.

5-HT_{2B} receptor

As previously mentioned, the 5-HT_{2B} receptor was originally described in the rat stomach fundus mediating contractile response to 5-HT. First cloned in 1992 the presence of the 5-HT_{2B} receptor in the CNS was originally controversial (Kursar *et al.*, 1992). Initial reports of mRNA distribution failed to detect 5-HT_{2B} transcripts in the rat brain (Foguet *et al.*, 1992, Pompeiano *et al.*, 1994). Later, 5-HT_{2B}-like immunoreactivity was detected in the rat brain (Duxon *et al.*, 1997a). The study found reasonably focal distribution of the receptor in the cerebellum, lateral septum, dorsal hypothalamus and was particularly high in the medial amygdala.

5-HT_{2C} receptor

This member of the 5-HT_2 class has been cloned in the rat and human (Julius *et al.*, 1988, Saltzman *et al.*, 1991). The 5-HT_{2C} receptor is mainly distributed in the CNS with little peripheral expression. Autoradiography and in situ hybridization found high expression in areas of the cortex, limbic system (hippocampus, amygdala and nucleus accumbens) and basal ganglia (caudate nucleus and substantia nigra) as well

as lower levels in NTS, nucleus ambiguous and raphé nuclei (Molineaux *et al.*, 1989, Wright *et al.*, 1995). There is mostly good agreement between ligand binding and mRNA distribution with one exception (Mengod *et al.*, 1990a). In the lateral habenular nucleus displays high mRNA but low 5-HT_{2C} binding sites suggesting it is one of the few locations were 5-HT_{2C} receptors are located pre synaptically.

Pharmacology

Importantly, for the use of these ligands in rat models, most 5-HT_2 ligands display very similar affinities for the human receptor. Most pharmacological characterization is performed on cloned human 5-HT_2 receptors in expression systems.
DOI is the classic agonist at all the 5-HT_2 class of receptors. It has a slightly higher potency the human 5-HT_{2A} receptor (pEC50 of 8.2, 7.5 and 7 at human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, respectively (Jerman *et al.*, 2001)). LSD is a partial agonist with a similar potency at all 5-HT_2 receptors (Porter *et al.*, 1999).
Overall the availability of agonists selective between the three 5-HT_2 sub-classes is poor; again Ro 60-0175 which considered to be selective for 5-HT_{2C} only shows a tenfold functional selectivity in the rat over 5-HT_{2A} receptors (Mbaki *et al.*, 2012). One ligand of interest is TCB-2, which is described as a potent agonist with high affinity at the 5-HT_{2A} receptor (McLean *et al.*, 2006). BW 723C86 is a commonly used 5-HT_{2B} agonist that does not cause stereotypical behavioural effects associated with 5-HT_{2C} receptor activation in rats (Kennett *et al.*, 1997).

Ketanserin is a widely used selective 5-HT_{2A} receptor antagonist but also has affinity for the 5-HT_{1D} receptor (Leysen *et al.*, 1981, Zgombick *et al.*, 1995) as well α_1-adrenoceptors and histamine H_1 receptors. MDL-100,907 is probably the most selective 5-HT_{2A} antagonist available being between 100 and 1000 fold more selective over 5-HT_{2C} and 5-HT_{2B} (Johnson *et al.*, 1996, Gleason *et al.*, 2001). 5-HT_{2B} receptor antagonists include RS-127445, which displays approximately 1000-fold selectivity for the 5-HT_{2B} receptor over other targets (Bonhaus *et al.*, 1999).

General roles in physiology

Activation of 5-HT_{2C} receptors causes membrane depolarization by reducing inward rectifying potassium currents and increases mobilization of intracellular calcium stores. Numerous electrophysiological studies have found 5-HT_{2A} and 5-HT_{2C}-mediated excitatory responses in CNS neurons. In one such example slices containing rat piriform cortex 5-HT caused depolarization of interneurons and pyramidal cells (Sheldon & Aghajanian, 1991). The highly selective 5-HT_{2A} receptor antagonist MDL-100,907 blocked depolarization of interneurons but the pyramidal cell was unaffected (Marek & Aghajanian, 1994). The authors concluded that the 5-HT_{2C} receptor depolarizes the pyramidal cells whereas 5-HT_{2A} depolarizes the interneurons. Hallucinogens have also been shown to be potent agonists in this preparation. LSD and DOI depolarize both pyramidal cells and interneurons of the piriform cortex (Marek & Aghajanian, 1996). Indeed, it is thought that these mechanisms mediate much of the hallucinogenic properties of these compounds.

Studies *in vivo* have shown 5-HT_{2A} receptor activation augments dopamine release in the rat nucleus accumbens caused by the reinforcing drugs cocaine, amphetamine and morphine (Porras *et al.*, 2002, Navailles *et al.*, 2008). It is thought to contribute to the rewarding properties of these drugs. Conversely, an earlier study, also *in vivo*, found a decrease in noradrenaline release in the hippocampus following the administration of DOI, which was reversed by non-selective 5-HT_2 antagonists (Done & Sharp, 1992). The authors concluded this was *via* an indirect inhibitory pathway. Such an inhibitory pathway has been previously described from the prepositus hypoglossal nucleus in the brainstem and also involves 5-HT_2 receptors (Gorea *et al.*, 1991). 5-HT_{2C} receptor antagonists increase the release of dopamine and noradrenaline in the frontal cortex when measured *in vivo* with microdialysis (Millan *et al.*, 1998). This data suggests that 5-HT_{2C} receptors are tonically active to inhibit both dopamine and noradrenaline release. Interestingly, the authors found no change in 5-HT concentration.

The behavioural effects of 5-$HT_{2A/2C}$ ligands have been well studied. Early studies reported systemic administration of 5-HT_2 agonists lead to a behavioural syndrome in rodents . In mice drugs in the DOx family, LSD, psilocybin and 5-HT releasing agents

cause head twitching and in rats 'wet-dog' shakes. Animals pre-treated with 5-HT_{2A} antagonists or 5-HT_{2A} knock-out mice no-longer display these stereotypical behaviours (Fiorella *et al.*, 1995, Gonzalez-Maeso *et al.*, 2007). 5-HT_{2A} agonists model many of the positive symptoms of schizophrenia such visual/auditory hallucinations and delusions. This, coupled with the fact that some antipsychotic drugs such as clozapine and risperidone are high-affinity 5-HT_{2A} antagonists has argued a role for 5-HT_{2A} in psychosis (see Gonzalez-Maeso & Sealfon, 2009).

The role of the 5-HT_{2B} receptors in the CNS is poorly understood. Low-density expression and poor selectivity of ligands have hampered research into their physiological role. However, the 5-HT_{2B} agonist BW-723C86 has been reported to have anxiolytic properties when administered systemically (Kennett *et al.*, 1998) and directly in to the medial amygdala (Duxon *et al.*, 1997b).

Behavioural effects of the 5-$HT_{2A/2C}$ receptors were initially difficult to distinguish from one another due to lack of selective ligands. 5-HT_{2C} receptor agonists also produce stereotyped behaviours in rodents such as hypolocomotion, hypophagia, anxiety, penile erections and hyperthermia (see Koek *et al.*, 1992). Hypophagia is of clinical interest as a target for anti-obesity. Indeed, lorcaserin (a 5-HT_{2C} agonist) has been recently approved for use in the treatment of obesity. Lorcaserin works by increasing satiety and therefore reducing food intake (see Carter *et al.*, 2012).

Role in autonomic function

5-HT_2 receptors act centrally and peripherally to regulate blood pressure. Peripherally they mediate vasoconstriction (see Kaumann & Levy, 2006). Centrally, 5-HT_{2A} activation causes sympathoexcitation and vasopressin release (see Ramage, 2001). Central application of the 5-HT_{2A} antagonist ketanserin caused no change in sympathetic activity in rat suggesting that these receptors were not tonically active, at least, in the anesthetised animal (Knowles & Ramage, 1999). However, infusion of MDL-100907 was shown to cause a fall in blood pressure (Mbaki *et al.*, 2012). 5-HT_{2B} activation with the selective 5-HT_{2B} agonist BW-723C86 causes a reduction in blood pressure (Knowles & Ramage, 2000). However, the selective 5-HT_{2B} receptor

antagonist RS-127445 did not change resting blood pressure in the anesthetised female rat suggesting no physiological role (Mbaki & Ramage, 2008).

Studies aimed at locating the 5-HT_{2A} receptors involved in this cardiovascular response found that application of the 5-HT_2 agonist DOI to the ventral surface of the brainstem caused a rise in blood pressure but no increase in heart rate. This effect was attenuated by i.v. propranolol and stellate ganglionectomy. These observations lead the authors to conclude that 5-HT_2 receptors at the level of the RVLM control a selective pathway that increase force but not rate of cardiac contractility (Mandal *et al*., 1990). However, a later study controlled the heart rate and blood pressure of the experimental animal artificially and found no change in cardiac contractility, only a change in peripheral vascular resistance (Ramage & Daly, 1998). It seems that 5-HT_2 receptors at the level of the RVLM affect sympathetic premotor neurons controlling only hind limb vascular resistance.

5-HT_2 receptors have also been implicated the micturition reflex (see Ramage, 2006). Indeed, spinal 5-$HT_{2A/2C}$ receptors were found to activate the external urethral sphincter (EUS) and the micturition reflex. On the other hand, central activation of 5-HT_{2C} receptor has been shown to inhibit the micturition reflex in anesthetised female rats but antagonists of this receptor alone had no effect, suggesting no physiological role (Mbaki & Ramage, 2008). A later study, where drug administration was optimized to provide selective and effective doses, supported the view of no physiological role in EUS activity or micturition reflex (Mbaki *et al.*, 2012).

Neurons in the NTS are known to express both 5-HT_{2A} and 5-HT_{2C} so they could both play a role in autonomic regulation. In this respect, when DOI was applied to neurons receiving vagal input it produced mixed excitation and inhibition (Wang *et al.*, 1997). Later, it was found that second-order neurons tended to be inhibited via 5-HT_{2C} activation and higher-order neurons were excited by 5-$HT_{2A/2B}$ receptors (Sevoz-Couche *et al.*, 2000). Further, when DOI was microinjected into the NTS producing a depressor response and bradycardia was completely inhibited by the selective 5-HT_{2A} antagonist MDL-100907, but not by 5-HT_{2B} and/or 5-HT_{2C} receptor agonists. (Comet *et al.*, 2007). The authors concluded that activation of the 5-HT_{2A} receptor alone in the NTS mediates the cardiovascular responses produced by non-selective 5-HT_2 agonists.

In vitro studies have found a role for 5-HT_2 receptors modulating neuronal excitability at the level of the NTS. One study found that the 5-HT_{2A} receptor antagonist ketanserin blocked the decrease in amplitude of EPSCs evoked by 5-HT (Takenaka *et al.*, 2011). 5-HT increased the paired-pulse ratio leading the authors to conclude that 5-HT was acting presynaptically on 5-HT_{2A} receptors to produce the inhibition. More recently, a study using multiple approaches has identified the presence of 5-HT_{2C} receptors that are tonically active, at least in the NTS slice preparation. 5-HT_{2C} receptor protein and transcripts were found in the NTS using real-time RT-PCR and immunohistochemistry in a post-synaptic location. Electrophysiological investigation using the selective 5-HT_{2C} receptor agonist CP-809101 augmented postsynaptic currents elicited by stimulation of the solitary tract and the 5-HT_{2C} antagonist RS-102221 inhibited these currents (Austgen *et al.*, 2012). Interestingly, RS-102221 reduced amplitude, but not frequency, of spontaneous mEPSCs suggesting that postsynaptic 5-HT_{2C} receptors are tonically activated by spontaneously released 5-HT.

1.4.5. 5-HT_3 receptors

The 5-HT_3 receptor was one of the first classes of 5-HT receptor to be reported (Gaddum & Picarelli, 1957). They were first reported as 5-HT 'M' receptors but later re-classified as the 5-HT_3 receptor.

Molecular biology

Unique within the 5-HT receptors as the only ligand-gated ion channel (Derkach *et al.*, 1989), the 5-HT_3 receptor belongs to a family known as the Cys-loop receptors. It is comprised of a pentamer of identical or non-identical subunits arranged to surround a water-filled pore in the centre of the protein. Each subunit has a large extracellular domain that forms the ligand-binding site (Boess *et al.*, 1992, Green *et al.*, 1995). There is significant structural homology between the 5-HT_3 and α7- nACh receptor to the extent that chimeric receptors are functional (Eisele *et al.*, 1993). Presently, five

distinct 5-HT_3 receptor subunits (A-E) have been identified, although C-E have are not found to be present in rodents (Holbrook *et al.*, 2009). Subunit combinations confer different ion channel properties; for example a receptor formed exclusively of 5-HT_3A subunits have lower single-channel conductance than when a 5-HT_3B subunit is incorporated (Gill *et al.*, 1995). It is also prone to rapid desensitization.

The ion pore is selectively permeable to Na^+ and K^+ and when activated causes ion influx resulting in fast membrane depolarization. There is some evidence of calcium permeability. Co-assembly of 5-HT_3 and α7-nAch receptor subunits produce Ca^{2+} permeable channels (van Hooft *et al.*, 1998). Later, evidence of 5-HT_3 Ca^{2+} permeability has been reported in hippocampal slices (Van Hooft & Wadman, 2003). The authors estimated that at physiological membrane potential (-60mV) the percentage of charge carried by Ca^{2+} was 0.1%. This is very low in comparison to NMDA receptors where ~18% of charge is carried by Ca^{2+} (Schneggenburger, 1996).

Distribution

In the PNS 5-HT_3 receptors are known to be present on the terminals of many sensory afferents. 5-HT_3 are thought to be responsible for the peripheral transduction of chemically-induced pain (Giordano & Dyche, 1989). When treated with capsaicin 5-HT_3 sites in dorsal horn of the rat spinal cord were found to be reduced (Hamon *et al.*, 1989). This observation suggests that 5-HT_3 receptors are located on C-fibre terminals. High densities are also found in autonomic nerves, especially the nodose ganglia and the vagus nerve in the cat and rabbit (Hoyer *et al.*, 1989).

The presence of 5-HT_3 receptors in the rat brain was first reported with the use of radiolabelled GR-65630 (Kilpatrick *et al.*, 1987). The highest density was found to be in the brainstem with NTS, DVN and area postrema showing particularly high levels of expression (Tecott *et al.*, 1993, Miquel *et al.*, 2002). The receptor is also found in the hippocampus, amygdala and regions of the cortex, albeit in lower concentrations and virtually no binding in pons, cerebellum, or basal ganglia (Waeber *et al.*, 1989, Barnes *et al.*, 1990).

Pharmacology

Ligands available for the 5-HT_3 receptor display good selectivity, which is most likely explained by the low sequence homology with other 5-HT receptor sub-types. Phenylbiguanide (PBG) is the archetypal full agonist for the 5-HT_3 receptor. However, it should be noted that there are interspecies differences with the potency of this agonist. Some 5-HT_3 ligands, including PBG, displays low affinity for the guinea pig receptor (Lankiewicz *et al.*, 1998). The closely related compound mCPBG is another highly selective agonist a the 5-HT_3 receptor with higher affinity than PBG (Kilpatrick *et al.*, 1990). There are also many selective antagonists available; granisetron and ondansetron are most commonly used and display high affinity for the 5-HT_3 with little or no affinity to other 5-HT receptors or other binding sites (Sanger & Nelson, 1989, Youssefyeh *et al.*, 1992). Volatile anaesthetics such as halothane and isoflurane, as well as alcohols, can allosterically modulate 5-HT_3 receptors (see Davies, 2011). Ethanol is known to stabilize the open time of these receptors leading to increased conductance (Feinberg-Zadek & Davies, 2010).

General roles in physiology

The primary clinical application for 5-HT_3 receptor ligands is as anti-emetics. 5-HT_3 receptor antagonists ondansetron, granisetron and, recently, palonosetron are all used to control emesis associated with drugs used in chemotherapy (Navari & Province, 2006). These drugs are thought to work at the level of the NTS and/or area postrema but peripheral 5-HT_3 receptors could be involved given the levels of expression in the gut (Glatzle *et al.*, 2002). Secondly, 5-HT_3 antagonists are routinely used for the relief of irritable bowel syndrome (see Gershon, 2004).

Peripherally, the presence of 5-HT_3 receptors on sensory afferents indicated a role in transduction of pain. Indeed, pain caused intradermal application of 5-HT is mediated by 5-HT_3 receptors (see Gershon, 2004). Later, studies have shown that genetic deletion of the 5-HT_3 receptor reduce responses to persistent pain-states after tissue

injury, such a formalin-induced inflammatory pain (Richardson *et al.*, 1985). There is also *in vivo* electrophysiological evidence of 5-HT_3 receptors facilitating transduction of noxious stimuli in the dorsal and ventral horn of the spinal cord (Zeitz *et al.*, 2002, Kayser *et al.*, 2007).

Central actions of 5-HT_3 receptors are wide ranging and have been implicated in several physiological processes and pathological states. 5-HT_3 antagonists and genetic deletion of the 5-HT_3 receptor has antinociceptive effect in the mouse (Rahman *et al.*, 2004, Asante & Dickenson, 2010). The amygdala is proposed to mediate these effects as microinjection of 5-HT_3 agonists into this area have found to have anxiogenic and antagonists have anxiolytic effects in rodents (Barnes *et al.*, 1992, Kelley *et al.*, 2003, Bhatnagar *et al.*, 2004).

5-HT_3 receptors have also been implicated in the control release of neurotransmitters acetylcholine and dopamine. Acetylcholine release is under tonic inhibitory control of 5-HT *via* 5-HT_3 receptors in human and rat cerebral cortex (Higgins *et al.*, 1991). Due to this effect, 5-HT_3 antagonists have been reported to have cognitive enhancing effects and have potential for therapeutic application in Alzheimer's disease (Maura *et al.*, 1992, Crespi *et al.*, 1997). With respect to dopamine, 5-HT_3 activation causes an increase in dopamine efflux in rat brain slices containing striatum (Fakhfouri *et al.*, 2012) and nucleus accumbens *in vivo* (Blandina *et al.*, 1989). Further, 5-HT_3 antagonists reduced the increase in dopamine efflux in the nucleus accumbens induced by nicotine, ethanol and morphine (Chen *et al.*, 1991). Ondansetron is known to reduced alcohol craving in some alcoholics, presumably by the same mechanism (Carboni *et al.*, 1989).

Role in autonomic control

5-HT_3 receptor ligands are not known to have any direct effect on vasculature or the heart, with the exception of dogs where cardiac 5-HT_3 receptor activation causes tachycardia (Johnson *et al.*, 2000). Activation of 5-HT_3 receptors located on vagal afferent terminals in the lungs and the heart cause bradycardia and hypotension. This

is known as the cardiopulmonary or von Bezold-Jarisch reflex that causes sympathetic withdrawal and an increase in vagal drive (see Ramage & Villalon, 2008).

Centrally, several brainstem nuclei display a particularly high density of 5-HT_3 receptors, suggesting an important role in autonomic regulation. Within this area 5-HT_3 receptors modulating glutamate release seem to account for most of their central autonomic functions. Iontophoretic application of PBG to vagal preganglionic neurons in the dorsal vagal nucleus causes excitation that was blocked by glutamate receptor antagonists (Wang *et al.*, 1996). This suggests that activation of presynaptic 5-HT_3 receptors augments glutamate release. A similar study in the NTS also found PBG increased excitability by increasing presynaptic glutamate release (Jeggo *et al.*, 2005). Consistent with this, 5-HT_3 antagonist granisetron attenuated the cardiopulmonary reflex when microinjected in the NTS (Pires *et al.*, 1998). Also consistent with 5-HT_3 receptors controlling glutamate release in the NTS, using *in vivo* microdialysis, extracellular glutamate was found to increase in response to local infusion of PBG (Ashworth-Preece *et al.*, 1995). However, there have been some reports showing that activation of the 5-HT_3 receptors in the NTS also cause attenuation of the bradycardia associated with the baroreflex, cardiopulmonary reflex and chemoreflex (Merahi *et al.*, 1992, Sevoz *et al.*, 1996, Sevoz *et al.*, 1997). The effects on the baroreflex and the chemoreflex were attenuated by bicuculline suggesting 5-HT_3 activation can cause inhibition *via* a GABAergic mechanism.

Data from *in vitro* slice preparations also confirm an excitatory role of 5-HT_3 receptors modulating glutamate release in the NTS. The first report of the properties of 5-HT_3 activation in the NTS showed both pre and postsynaptic effects. The authors showed that application of 5-HT and 2-CH_3-5-HT caused depolarisation even in the presence of glutamate antagonists and when release was blocked with cobalt (Glaum *et al.*, 1992). Later, it was shown that presynaptic 5-HT_3 receptors were tonically active in the NTS slice as glutamate release decreased in the presence of 5-HT_3 antagonist ondansetron (Wan & Browning, 2008). Interestingly, this effect was abolished by vagotomy suggesting vagal afferent terminals expressed the 5-HT_3 receptors and/or release 5-HT. Conversely, 5-HT_3 activation has been shown to have inhibitory effects in the rat and mouse NTS slice (Takenaka *et al.*, 2011, Cui *et al.*, 2012). When stimulating the solitary tract the resultant EPSP amplitude was reduced

in the presence of 5-HT_3 agonists 2-CH_3-5-HT and SR-57227, this effect was antagonised by 5-HT_3 blockade. Both studies found that 5-HT_3 activation caused large increases in spontaneous release of glutamate so it seems 5-HT_3 has two distinct modulatory effects. The latter study theorised that the increase in spontaneous activity depletes the releasable pool of glutamate reducing that available to be released with stimulated.

1.4.6. 5-HT_4, 5-ht_5 and 5-HT_6 receptors

5-HT_4 receptors

First reported in 1988, 5-HT_4 receptors showed a distinct pharmacological profile to other 5-HT receptors positively coupled to adenylyl cyclase. The human 5-HT_4 gene has ten functional spice variants reported to date but all display similar pharmacology (Dumuis *et al.*, 1988).

Distributed peripherally mainly in the gut and heart where they have been found to mediate contractility of smooth muscle. In the CNS 5-HT_4 receptors are mainly found postsynaptically in limbic areas, with maximal expression in the basal ganglia (Bockaert *et al.*, 2008).

Selective ligands are available and include agonists BIMU 8 (potent full-agonist), tegaserod (partial agonist) and zacopride (mixed 5-HT_4 agonist/ 5-HT_3 antagonist). Selective, high-affinity antagonists include GR-113808, SB-204070 and RS-100235.

5-HT_4 receptors function to modulate the release of several neurotransmitters and enhance synaptic transmission. Strong roles in learning and memory have been proposed as many studies have shown cognitive improvement associated with increased 5-HT_4 activation (Varnas *et al.*, 2003).

5-ht_5 receptors

Despite being discovered more than 20 years ago the 5-ht_5 still exists as only a gene product. In order to signify the lack of identified physiological roles the abbreviation is written in lower case. Two sub-types have been identified (5-$ht_{5a/b}$), although the 5-ht_{5b} gene is likely to encode a non-functioning protein in humans, the rodent gene does appear to encode a functional protein. The method of signal transduction for this receptor is unknown with conflicting reports in expression systems of possible inhibition of adenylyl cyclase, presumably *via* G_i (Bockaert *et al.*, 2004).

Distribution of the receptor is unclear due to lack of selective ligands precluding autoradiography. 5-ht_5 mRNA in the human brain was found cortex, hippocampus, amygdala and cerebellum (Francken *et al.*, 1998, Grailhe *et al.*, 2001).

Selective ligands are non-existent (see Ramage, 2004) but 5-CT is a 5-ht_5 agonist and SB-699551-A is a putative antagonist with a 30-fold selectivity for human 5-HT_{5A} but has affinity to 5-HT transporter at 10 times higher concentrations (Pasqualetti *et al.*, 1998).

Possible roles have been suggested in regulation of circadian rhythm, however lack of selective ligands makes interpretation difficult (Thomas, 2006). Further, genetic deletion of 5-ht_{5A} receptors in mice display increased exploratory activity and altered response to LSD, which is a 5-ht_{5A} ligand (Sprouse *et al.*, 2004).

5-HT_6 receptors

Originally cloned in rat, the 5-HT_6 receptor is a G-protein coupled receptor that activates adenylyl cyclase (Grailhe *et al.*, 1999). There is only one functional isoform of this receptor produced. *In situ* hybridization of 5-HT_6 mRNA in the rat brain reveals a distribution confined to the CNS. In order of decreasing density; olfactory tubercle, entorhinal cortex, dentate gyrus, hypothalamus, amygdala and substantia nigra were all found to contain 5-HT_6 transcripts (Ruat *et al.*, 1993a). Later,

immunohistochemistry and radioligand binding were used to confirm this distribution (Gerard *et al.*, 1997).

There has been a range of high affinity ligands developed for the 5-HT_6 receptor. Agonists include EMDT and EMD-386088 which are reasonably selective for 5-HT_6 receptors. Recently, ST-1936 has been reported as a high affinity, selective agonist (Hamon *et al.*, 1999). Highly selective antagonists are also available which include Ro 04-6790 and SB-271046. Many clinically used antipsychotic and antidepressant drugs also share affinity with the 5-HT_6 receptor, for example clozapine and imipramine (Riccioni *et al.*, 2011).

5-HT_6 receptor have been shown to be involved in many animal models of cognitive function (Monsma *et al.*, 1993). 5-HT_6 antagonists have been shown to enhance the release of monoamines, glutamate and acetylcholine that can be associated with improvements in memory function (Fone, 2008). This has generated a significant amount of interest for 5-HT_6 antagonists in the treatment of Alzheimer's disease and some have entered clinical trials (Lacroix *et al.*, 2004, Kendall *et al.*, 2011).

1.4.7. 5-HT_7 receptors

The 5-HT_7 receptor is the most recently identified class in the 5-HT receptor family. First reported as an unknown 5-HT receptor active in the vena cava of the swine (Trevethick *et al.*, 1984). It was later found to be positively coupled to adenylyl cyclase and activated by 5-CT (Trevethick *et al.*, 1986). This novel pharmacology distinguished it from other identified 5-HT receptors at the time. Later, three independent groups cloned the receptor from the rat and human, confirming the existence of a separate class of 5-HT receptor (Sumner *et al.*, 1989). Since then it has been cloned in a wide variety of species including mouse, guinea pig, *C. elegans, Xenopus laevis* and honeybee (Bard *et al.*, 1993, Lovenberg *et al.*, 1993, Ruat *et al.*, 1993b).

Molecular Biology

The gene encoding the 5-HT_7 is found on the chromosome 10q23.3-q24.4 and contains three introns (Matthys *et al.*, 2011). Splice variants from second and third introns have been found to generate 5 receptor isoforms, 5-$HT_{7a–e}$ (Heidmann *et al.*, 1997). All isoforms are functionally active and no differences in pharmacology or signal transduction properties have been reported (Liu *et al.*, 2001). Receptor isoforms differences are all located in the C-terminus of the receptor possibly regulating trafficking (Heidmann *et al.*, 1998, Krobert *et al.*, 2001)

Distribution

The 5-HT_7 receptor has been found to be expressed in the CNS, PNS and in peripheral tissues. In the CNS autoradiography with [^{3}H]-5-CT combined with *in situ* hybridization found strongest binding in the lateral/medial septum, amygdala and some areas of thalamus. There were lower levels found in cortex, hippocampus and hypothalamus and substantia nigra. Low binding was also observed in dorsal raphé, periaqueductal grey, pontine nuclei, NTS, and dorsal horn of the spinal cord (Guthrie *et al.*, 2005). Further, one study multiple approaches were used to show the presence of 5-HT_7 receptors in the CNS (Gustafson *et al.*, 1996). The authors used c-fos expression induced by 8-OH-DPAT in the presence of WAY-100635 coupled with a 5-HT_7 antibody to double-label 5-HT_7 expressing neurons. This approach greatly reduced the possibility of immuno-cross reactivity giving rise to false-positive labeling. 5-HT_7 immunoreactivity was found in cortex, thalamus, hypothalamus, and hippocampus. This largely agreed with the earlier study. Expression of 5-HT_7 seems to be strongest in limbic areas but it is also expressed in those involved in central cardiovascular control, such as the NTS.

Pharmacology

Until recently, there has been paucity of selective 5-HT_7 agonists. Two compounds have been identified as putative 5-HT_7 selective agonists; LP 12 and LP 44 (Neumaier *et al.*, 2001). These compounds show >100-fold selectivity over 5-HT_{1A} receptor. Unfortunately little data exists from their use as selective 5-HT_7 agonists and both

compounds are poorly soluble in water. Most studies to date have used non-selective agonists 5-CT and 8-OH-DPAT. 5-Carboxamidotryptamine (5-CT) seems to have the highest affinity (pK_i 9.1 at human 5-$HT_{7(a)}$). 8-OH-DPAT also has moderate affinity with pK_i 6.6 at human 5-HT_7. For comparison, 5-HT itself displays a receptor affinity of 8.1. Unfortunately, both 5-CT and 8-OH-DPAT have agonist activity at 5-HT_{1A} and 5-CT also has additional agonist activity at 5-HT_{1B} and 5-HT_{1D} receptors. Indeed, many affects originally thought to be mediated by these receptors are now being re-assessed to 5-HT_7 mediated (Leopoldo *et al.*, 2004, Leopoldo *et al.*, 2007).

More recently, highly selective antagonists of the 5-HT_7 receptor have been reported. The first to display greater than 100-fold selectivity over other 5-HT receptors was SB-258719 (Eglen *et al.*, 1997). This compound has a pK_i of 7.5, antagonists with higher affinity are available but the water solubility of this compound makes it more convenient for certain experimental designs. Later, SB-269970 was developed with a higher affinity having a pK_i of 8.9 at human 5-$HT_{7(a)}$ receptors (Forbes *et al.*, 1998). However, as mentioned in section 1.4.7, SB-269970 has only a 50-fold selectivity over 5-HT_{5A} receptors (Lovell *et al.*, 2000).

General Roles in Physiology

Given the distribution of 5-HT_7 receptors in the supra-chiasmatic nucleus (SCN) it is unsurprising that one of the first identified roles is that in circadian rhythms. Sleep-wake cycle, body temperature, hormone release and behavioural patterns are all synchronized to the light/dark cycle by the SCN. The SCN receives 5-HT input from the dorsal raphé *via* the median raphé nucleus (Hagan *et al.*, 2000). 8-OH-DPAT induced a phase-shift in SCN neuronal activity that was antagonised by ritanserin (5-$HT_{2/7}$ antagonist) but not pindolol (Glass *et al.*, 2003). This was confirmed *in vivo* the more selective 5-HT_7 antagonist DR4004 (Lovenberg *et al.*, 1993).

5-HT_7 receptors expressed in the dorsal raphé have been shown to control the release of 5-HT in the SCN. Pharmacological and electrical stimulation of the dorsal raphé and median raphé nuclei caused increases in extracellular 5-HT concentration in the SCN, measured by *in vivo* microdialysis. Microinjection of the 5-HT_7 antagonist DR4004 in the dorsal raphé or median raphé reduced the efflux of 5-HT in the SCN

(Ehlen *et al.*, 2001). Thus, 5-HT_7 receptors control the SCN by regulating the supply of 5-HT and directly as it is expressed on cells within the SCN. It should, however, be noted that DR4004 has agonist activity at the D2 receptor (Glass *et al.*, 2003).

The suppression of 5-HT release was attenuated by bicuculline, which suggests that 5-HT_7 activation leads to inhibition of GABA interneurons. In turn, this reduces GABA release and reduces the inhibitory tone on 5-HT neurons. Immunohistochemical analysis also indicates that 5-HT_7 may be located on GABA interneurons and not the 5-HT-containing neurons themselves (Kogan *et al.*, 2002).

The role of 5-HT_7 in thermoregulation was used an effective screen for compounds with 5-HT_7 activity. 5-CT induced hypothermia in guinea pigs and was used to characterize SB-269970 as a selective 5-HT_7 antagonist (Duncan *et al.*, 2001). This role was confirmed in a comprehensive study using multiple drugs and 5-HT_7 knock out animals (Hagan *et al.*, 2000).

The possibility of 5-HT_7 receptors as a target for anti-depression was due to their ability to modify REM sleep patterns similar to those of known anti-depressant drugs. Genetic deletion or antagonism of the 5-HT_7 receptor was shown to increase antidepressant-like behavior in two models of depression, the forced swim test and tail suspension test (Guscott *et al.*, 2003). Recently, the use of 5-HT_7 antagonists as an adjunct to classical antidepressant drugs to reduce the clinical latency associated with these treatments. It was found that SB-269970 enhanced the effect of a number of monoamine re-uptake in the forced swim test (Hedlund *et al.*, 2005, Wesolowska *et al.*, 2006).

Role in Autonomic Control

Little was known regarding the role of 5-HT_7 receptor in central autonomic control due to a lack of selective ligands and the relatively late discovery of the receptor itself. 5-HT_7 is expressed in peripheral tissues such as vasculature and heart as well as brain nuclei involved in cardiovascular regulation.

5-HT_7 receptors are known to be expressed in vascular smooth muscle and when activated cause dilatation (Wesolowska *et al.*, 2007). Additional peripheral activity was found in the cat where activation 5-HT_7 on the heart caused tachycardia (Centurion *et al.*, 2004).

When administered centrally SB-269970 was found to have no effect on resting blood pressure. However, this compound was first found to inhibit the micturition reflex in anaesthetised rats (Read *et al.*, 2003) Further, it was also found to inhibit vagal bradycardia associated with baroreceptor, cardiopulmonary and chemoreceptor reflex activation in anaesthetised and unanaesthetised rats (Kellett *et al.*, 2005, Damaso *et al.*, 2007). The receptors involved were believed to be in the NTS and a later study reported evidence that 5-HT_7 receptors mediated transmission in the NTS (Oskutyte *et al.*, 2009).

1.5. Neurotransmitter reuptake

Background

The effect of all neurotransmitters is terminated by either removal from the site of action by uptake proteins or degradation to non-active compounds by enzymes. The neurotransmitter acetylcholine (ACh) is an example of termination by enzyme. This fast-acting transmitter requires an enzyme with an extremely high turnover rate to terminate the action of ACh. Indeed, acetylcholinesterase has one of the highest turnover rates of all enzymes known in mammalian physiology and is capable of converting ACh to choline and acetate at a rate of 25,000 per second (see Quinn, 1987). The choline is then transported back into cells *via* a high affinity plasmalemmal transporter where it can be recycled (Apparsundaram *et al.*, 2000). On the other hand, transmitters like glutamate, GABA, 5-HT, dopamine and noradrenaline are taken up into cells before being degraded (Masson *et al.*, 1999). The advantage here is that, once inside the presynaptic terminal the molecule can be re-packaged in vesicles ready for release. Experiments performed with tritiated adrenaline and noradrenaline found that when these were injected into the circulation

of experimental animals 30-40% was taken into tissues where it remained unchanged (Axelrod *et al.*, 1959, Whitby *et al.*, 1961). This concept of re-uptake was explored further in the brain first using slices of cortical tissue (Dengler *et al.*, 1961). The authors concluded that there was an active process concentrating noradrenaline into the tissue. Further, this uptake of noradrenaline was shown to be sensitive to extracellular sodium and chloride ion concentrations (White & Keen, 1970). These were some of the first reports of the high affinity, low capacity re-uptake system we now know as DAT (dopamine transporter; SLC6A3), NET (noradrenaline transporter; SLC6A2), SERT (5-HT transporter (5-HTT); SLC6A4). Another re-uptake system was discovered in the isolated rat heart when perfusion of tritiated noradrenaline resulted in grater than proportional increase in rates of uptake at higher concentrations (Iversen, 1965). This system was found to be insensitive to known re-uptake inhibitors and, as such, was named 'Uptake 2'. This was later found to be part of the OCT (SLC22) family of transporters. This constituted a low affinity but high capacity re-uptake system and is know to include PMAT (SLC24). It is important to note that PMAT is distinct to VMAT that serves to package monoamines into vesicles and is not directly responsible for removing them from the extracellular medium.

Once taken up into tissues monoamines are metabolized by catechol *O*-methyltransferase (COMT) and a family of similar enzymes known as monoamine oxidases (MAO). These enzymes breakdown all monoamines but these are primarily in the cytoplasm so do not terminate the action of monoamines at their receptor. However, there is some evidence that COMT in directly involved in the termination of dopamine in brain areas with low DAT expression (Matsumoto *et al.*, 2003).

1.5.1. 5-HT transporter (SLC6A4)

Molecular biology

The 5-HT transporter was first cloned in 1991 from an RNA that is associated with 5-HT-rich regions of the midbrain and brainstem (Blakely *et al.*, 1991). Upon expression, this 12 transmembrane domain protein was found to transport 5-HT *via* a

sodium dependent mechanism that is sensitive uptake inhibitors such as paroxetine, citalopram and fluoxetine. This transporter, like all others in the SLC6 family, is a co-transporter and uses the extracellular sodium gradient to drive uptake (Nelson, 1998). Sodium is absolutely required for transport to occur. Chloride and potassium ions are also co-transported with a predicted stoichiometry of 1 5-HT:1 Na^+:1Cl^- (in) and 1 K^+ (out) but their presence is not required for reuptake to occur (Talvenheimo *et al.*, 1983). These transporters can also operate in reverse if the intracellular concentration of 5-HT is increased and facilitate 5-HT efflux from the nerve terminal under certain conditions. Interestingly, the 5-HT transporter has been shown to internalize upon activation of PKC, which will decrease 5-HT re-uptake (Bauman *et al.*, 2000).

Distribution

The 5-HT transporter has a wide distribution in both brain and the periphery. Peripherally, 5-HT transporters are known to be expressed in platelets where they sequester 5-HT from the plasma (Sneddon, 1973). They are also present in areas with high concentrations of 5-HT such as pulmonary epithelia and enteric system.

Autoradiography with 5-HT re-uptake inhibitor [^{3}H]- citalopram showed dense binding in midbrain, hypothalamus and to a lesser extent in the medulla, hippocampus and lowest in the cerebellum (D'Amato *et al.*, 1987). However, this protocol displayed high (~30%) non-specific binding so resolution was poor. Later, one study optimized the binding conditions with [^{3}H]- paroxetine and found highest density binding in the dorsal raphé with decreasing levels in the locus coeruleus, substantia nigra, amygdala and hippocampus (Chen *et al.*, 1992). Immunohistochemistry revealed a similar distribution of 5-HT transporter protein binding (Qian *et al.*, 1995). There is a lack of binding data in the rat brainstem, especially the NTS. However, one study found the presence of the 5-HT transporter on axon terminals in the medial NTS (Huang *et al.*, 2004). Additionally, human studies found 5-HT transporter-like binding sites in the brain stem (Schain *et al.*, 2013).

Investigation into the subcellular location of the 5-HT transporter has found evidence of expression on axons as well as 5-HT-containing terminals (Zhou *et al.*, 1998). Further, using electron microscopy, evidence for low plasma membrane soma-dendrite expression of these transporters but high expression along the axon plasma membrane (Tao-Cheng & Zhou, 1999). These extrasynaptic 5-HT transporters support the view that 5-HT is also a volume transmitter.

5-HT transporter expression is thought to be almost exclusively neuronal in the brain with no evidence thus far of glial expression of any SLC6 monoamine transporter, unlike GABA and glycine transports in the same class (see Torres *et al.*, 2003, Kristensen *et al.*, 2011).

Pharmacology

The first drugs available as 5-HT transporter antagonists were tricyclic antidepressants (TCAs) were developed in the 1950's (see Andersen *et al.*, 2009). TCAs displayed poor selectivity over other SLC6 monoamine transporters and especially over the noradrenaline transporter (see table 1.1). Their widespread use in the treatment of affective disorders, such as depression, lead to intense research into more selective reuptake inhibitors. This paved the way for the development of so-called selective serotonin re-uptake inhibitors (SSRIs; Wong *et al.*, 1974). Despite their name, this drug class also prevents the re-uptake of noradrenaline and dopamine to a greater or lesser extent. Paroxetine has the highest potency at the 5-HT transporter but not the highest selectivity (table 1.1 and 1.2). Citalopram, while not the most potent, is the most selective for the 5-HT transporter over the noradrenaline transporter and dopamine transporter. Interestingly, fluoxetine is the least selective displaying significant affinities for the noradrenaline transporter. Re-uptake inhibitors are often used to confirm the identity of electrochemical signals but given the selectivity of the drugs available, their use should be carefully considered (Phillips & Wightman, 2003).

The main use of these drugs is in affective disorders such as depression, anxiety and post-traumatic stress disorder (see Andersen *et al.*, 2009). The biochemical actions

(increasing extracellular 5-HT) of these drugs are not directly linked with their clinical effectiveness. Indeed, initial increases in extracellular 5-HT will activate autoreceptors, especially in the dorsal raphé leading to a decrease in 5-HT release in the terminal fields (see Artigas *et al.*, 1996). Additionally, whereas the pharmacological effect of these drugs occurs in matter of minutes clinical latency is at least three weeks. This suggests additional mechanisms underlying their clinical action which have been extensively reviewed elsewhere (Castren, 2005).

Table 1.1 Pharmacological profiles of re-uptake inhibitors

Comparison of inhibition constants at cloned human 5-HT, noradrenaline and dopamine transporters of a range of monoamine transporter antagonists. Compounds are ranked according to potency at the human 5-HT transporter.

	Compound	K_D (nM)[a]		
		h5-HTT	hNET	hDAT
Highest	Paroxetine	0.13	40	490
	Clomipramine	0.28	38	2190
	Sertraline	0.29	420	25
	Fluoxetine	0.81	240	3600
	Duloxetine	1	8	240
	Citalopram	1.16	4070	28100
	Imipramine	1.4	37	8500
	Fluvoxamine	2.2	1300	9200
	Desipramine	17.6	0.83	3190
	Reboxetine	242[b]	3[b]	>10000[b]
	Cocaine	340	1420	220
Lowest	GBR12909	>100	>100	1[c]

Values taken from (Tatsumi *et al.*, 1997) unless otherwise stated, [b](Deecher *et al.*, 2006), [c]data from rat (Andersen, 1989).

Table 1.2 Selectivity of monoamine re-uptake inhibitors

Comparison of the selectivity of a range of monoamine reuptake inhibitors from Table 1.1. Compounds are ranked according to selectivity at the 5-HT transporter over the noradrenaline transporter.

	Compound	Selectivity Quotient[a]		
		Ratio 5-HTT:NET	Ratio 5-HTT:DAT	Ratio NET:5-HTT
Highest	Citalopram	3508.62	24224.1	0.0003
	Sertraline	1448.28	86.2	0.0007
	Fluvoxamine	590.91	4181.8	0.0017
	Paroxetine	307.69	3769.2	0.0033
	Fluoxetine	296.30	4444.4	0.0034
	Clomipramine	135.71	7821.4	0.0074
	Imipramine	26.43	6071.4	0.04
	Duloxetine	8.00	240.0	0.13
	Cocaine	4.18	0.6	0.24
	Desipramine	0.05	181.3	21.21
	Reboxetine	0.01	N/A	80.67
Lowest	GBR12909	N/A	N/A	N/A

[a]Selectivity quotient is calculated as the ratio of the K_D's for each compound at each transporter.

Role in autonomic control

Their effect on central autonomic control have not been extensively investigated. The effects observed are consistent with the acute effect of re-uptake inhibitors on transmitters involved in autonomic reflexes. The monoamine re-uptake inhibitor fluoxetine was found to potentiate bronchoconstriction, which is facilitated by 5-HT_{1A} activation, when applied centrally to the guinea pig (Bootle *et al.*, 1998). Further, baroreflex enhancement after repeated fluoxetine administration was reported in rats (Moffitt & Johnson, 2004). Another more recent study examined chronic fluoxetine treatment in an animal model of chronic heart failure (Henze *et al.*, 2013). Autonomic changes were found in rats that received sham surgery indicating fluoxetine treatment alone causes reduced sympathetic and vagal drive. In animals with chronic heart failure fluoxetine compounded the already reduced control of normal sinus rhythm, possibly by desensitization of cardiac β-receptors. The latter effect could be explained by peripheral noradrenaline re-uptake inhibition.

In humans fluoxetine rectified the decreased heart rate variability seen in patients with post-traumatic stress disorder (Cohen *et al.*, 2000). Decreased heart rate variability indicates increased sympathetic tone, which is consistent with increased feelings of stress. It remains to be determined whether fluoxetine had a direct effect on sympathetic tone or indirectly by reducing stress-induced sympathoexcitation. Alternatively, no differences in autonomic variables were found when compared to depressed patients on two classes of monoamine reuptake inhibitors (Sattler *et al.*, 2000) and after sertraline administration in healthy volunteers (Ahrens *et al.*, 2007). The noradrenaline re-uptake inhibitor reboxetine was found to increase heart rate in human subjects however reboxetine has anti-muscarinic activity that could account for this effect (Penttila *et al.*, 2001).

1.5.2. Organic cation transporter

Molecular biology

The presence of 'Uptake 2' in the cloned cell line Caki-1 derived from human renal cell carcinoma was the first step in the molecular characterization of this class of transporters (Schomig & Schonfeld, 1990). The authors were the first to draw a parallel between the previously identified 'Uptake 2' in cardiac tissue and an organic cation transport system in the kidney. Later, this organic cation transporter was cloned from the rat kidney (OCT1; Grundemann *et al.*, 1994). Since the cloning of rOCT1 two additional isoforms of the organic cation transporter have been discovered; OCT2 and OCT3 (Okuda *et al.*, 1996, Grundemann *et al.*, 1998). These transporters are part of the SLC22 transporter family (see Koepsell & Endou, 2004). OCTs are 500-600-amino acid membrane proteins with a predicted 12 transmembrane spanning domains, a large glycosylated extracellular loop and a large intracellular loop. Both loops have sites for glycosylation and phosphorylation possibly used for regulation. Transport is known to be independent from sodium concentration, electrogenic and driven solely by the electrochemical gradient of the substrate (see Ciarimboli & Schlatter, 2005).

Distribution

OCT1 and 2 are mainly expressed in the periphery. OCT1 is found in the liver of rat, mouse rabbit and human. Additionally, rodent OCT1 is found in kidney, small intestine, colon, skin and spleen (see Koepsell & Endou, 2004). OCT2 in rats and humans has been found to be mainly expressed in the kidney on the proximal tubules (Karbach *et al.*, 2000, Motohashi *et al.*, 2002). OCT3, on the other hand, has a wide distribution including heart, liver, kidney and CNS (Wu *et al.*, 1998). OCT3 was formally known as extraneuronal monoamine transporter due to original discovery transporting neurotransmitters in non-neuronal tissues. However, subsequent investigation has found OCT3 expression on neuronal tissue (Verhaagh *et al.*, 1999). Central expression of OCT3 is highest in areas rich in monoamines. OCT 3 is the primary OCT expressed in the brain and *in situ* hybridization found mRNA the

cerebellum, dorsal raphé, hypothalamic nuclei, cortex and hippocampus (Amphoux *et al.*, 2006). In mice OCT3 mRNA has been found exclusively in neuronal cells types in the hippocampus and cerebellum, other areas were not investigated (Schmitt *et al.*, 2003). Interestingly, the authors found that OCT3 mRNA was up-regulated in 5-HT transporter knock-out animals supporting overlapping roles of the two transporters. However, immunohistochemistry has found OCT3 expressed on glial-like structures in the dorsomedial hypothalamic nucleus and striatum of the rat (Gasser *et al.*, 2006, Cui *et al.*, 2009).

Pharmacology

Most of the following data is from cloned human OCTs expressed in heterologous expression systems. OCT sequence homology is approximately 80% across species so binding data is expected to be comparable in rat models (see Shu, 2011).

OCT3 has a pharmacological profile distinct to that of 5-HTT, DAT and NET. OCT3 is insensitive to monoamine re-uptake inhibitors such fluoxetine, citalopram. However, TCA's such as desipramine and imipramine have been shown inhibit MPP+ reuptake *via* OCT3 at concentrations above 10μM in human retinal pigment epithelial cells (Wu *et al.*, 2000). OCT3 is often defined by its sensitivity to steroids. Indeed, corticosterone, progesterone and β-estradiol have all been shown to be inhibitors in the low μM-range. Corticosterone is the most potent at OCT3 with IC_{50} 0.29μM followed by β-estradiol (2.88) and progesterone (4.28) measured by inhibition of MPP+ uptake by OCT3 expressed in HEK cells (Hayer-Zillgen *et al.*, 2002). The most potent inhibitors of OCT3 belong to a class of compounds known as isocyanines. The first reported is decynium-22 (D-22) with an IC_{50} 90nM measured by inhibition of MPP+ uptake by OCT3 expressed in HEK cells (Schomig *et al.*, 1993, Hayer-Zillgen *et al.*, 2002). D-22 is the most commonly used inhibitor of OCT3, however there is little data regarding selectivity of these compounds. There is evidence that D-22 is also a α_1-adrenoceptor antagonist as it lowers blood pressure in anesthetised rabbits (Russ *et al.*, 1996). The same study found that D-22 has a relatively short half-life of approximately 9 minutes. Interestingly, D-22 was also shown inhibit baroreflex responses to this hypotension possibly by blockade of central α_1 receptors as D-22 has little affinity for the β- adrenoceptor (Konigs *et al.*, 1996).

Interestingly, some drugs of abuse are known to block OCT3 including MDMA, ketamine and phencyclidine (Amphoux *et al.*, 2006) but not amphetamine (Zhu *et al.*, 2010).

Function

OCTs have a wide range of substrates both synthetic and endogenous (for references see Shu, 2011). TEA and MPP+ are readily transported substrates of OCTs and have been used to characterise these transporters. Some clinically used drugs have also found to be substrates such as metformin, pindolol and propranolol. Endogenous substrates include dopamine, adrenaline, noradrenaline and histamine. These transports display low affinity (K_m) for the endogenous substrates, however they have extremely high transport rates (V_{max}) making them efficient at removing high concentrations of these neurotransmitters from the extracellular medium, particularly OCT3 (Amphoux *et al.*, 2006).

Within the brain these transporters have been proposed to work in concert with the high-affinity transporters 5-HTT, NET and DAT allowing control of extracellular monoamines over a wide range of concentrations (Gasser *et al.*, 2009, Duan & Wang, 2010). A scheme for the 5-HT clearance has been proposed that incorporates high-affinity monoamine transporters and OCT3 (Daws, 2009). At low concentrations (nM) of 5-HT, the 5-HTT provides the primary means of clearance, while at higher concentrations OCT3 takes over (see figure 1.4). Also of note is the possibility of other transporters capable of removing 5-HT at high concentration, so-called promiscuous transport. In this respect it has been shown, under certain conditions, that DAT and NET can transport 5-HT from the extracellular medium (Stamford *et al.*, 1990, Daws *et al.*, 1998). From this, it is seems that OCT mediated clearance of these transmitters occurs only when concentration are high for example during periods of intense stimulation (Bunin & Wightman, 1998) or when the high-affinity transport has been compromised by drugs or genetic deletion (Baganz *et al.*, 2008, Wultsch *et al.*, 2009, Daws *et al.*, 2013). However, synaptic concentrations of 5-HT have been calculated to be ~6μM, which was sufficient to saturate 5-HTT (Daws, 2009) so OCTs could be responsible for removal of 5-HT under normal physiological conditions.

OCT3 in the brain may play a role in volume or 'paracrine' transmission mediated by monoamines. Unlike traditional point-to point transmission where the pre-synaptic terminal is closely apposed to the post-synaptic receptors volume transmission is not confined to the synaptic cleft (see Bunin & Wightman, 1999, Fuxe *et al.*, 2005). OCT3 may control the spread of monoamines outside the synaptic cleft when the capacity of the high-affinity transporters is exceeded.

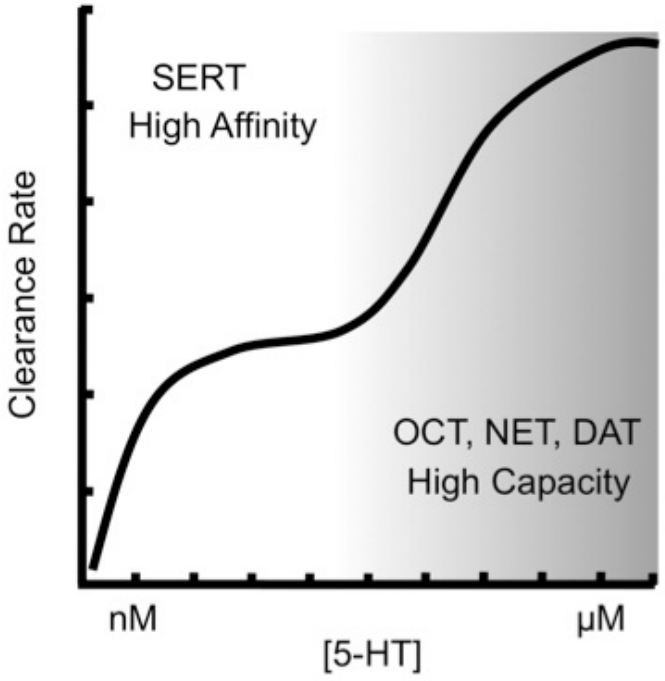


Figure 1.4 5-HT clearance by monoamine transporters

Predicted clearance rate of 5-HT from the extracellular medium against concentration. The function is polynomial due to the presence of two uptake systems; one high affinity, low capacity and one low affinity but high capacity (Bunin & Wightman, 1998).

1.5.3. Plasma membrane monoamine transporter (PMAT; SLC29A4)

First cloned in 2004 from the human brain PMAT was shown to be a low-affinity but high capacity transporter of monoamines (Engel *et al.*, 2004, Engel & Wang, 2005). PMAT is identified as a sodium-independent, electrogenic cation transporter with a wide range of substrates. The protein contains 530 amino acids with 10-12 transmembrane domains, very similar to the OCT family. Similarly, both PMAT and OCTs are sensitive to decynium-22 blockade (Hayer-Zillgen *et al.*, 2002, Engel *et al.*, 2004). However, the same studies found that PMAT was insensitive to the steroid corticosterone, unlike OCTs (Engel & Wang, 2005). Direct comparison of transport efficiency (V_{max}/K_m) between human OCT3 and PMAT expressed in HEK cells found that 5-HT and dopamine were preferentially taken up by PMAT. Alternatively, OCT3 has a higher preference for histamine, noradrenaline and adrenaline over other biogenic amines (Duan & Wang, 2010). The same study found that PMAT was insensitive to inhibitors of high-affinity monoamine transporters such as citalopram, GBR-12909 and desipramine. An earlier study substantiated this by showing a range of antidepressant and antipsychotic drugs had IC_{50}'s of 10-20μM at the human PMAT (Haenisch & Bonisch, 2010).

PMAT distribution has been investigated in mouse and rat brain (Dahlin *et al.*, 2007, Vialou *et al.*, 2007). The authors reported PMAT to be widely distributed throughout the brain and not necessary associated with monoamine pathways, indeed it was completely absent from the rat substantia nigra. Significant expression in the rat brainstem was found with the NTS, DVN and hypoglossal nucleus displaying high immunoreactivity. Expression of PMAT has been reported to be primarily neuronal. Cells double-labeled with glial fibrillary acidic protein and PMAT were not observed in any great number in the mouse brain (Dahlin *et al.*, 2007). However, there is evidence PMAT expression in cultured human astrocytes where they mediate histamine transport (Yoshikawa *et al.*, 2013).

1.6. The autonomic nervous system

1.6.1. The nucleus tractus solitarus (NTS)

Background

The nucleus responsible for orchestrating the cardiovascular reflexes is the NTS and is the site at which a range of visceral afferent fibres terminate. This nucleus is understood to play a central role in autonomic function. One interesting clinical example illustrating this role has been described (Biaggioni *et al.*, 1994). This case report describes a patient presenting with a complete absence of tachycardia in response to hypotension caused by i.v. nitroprusside. Post-mortem histological analysis of the patient's brainstem showed bilateral damage to the NTS alone.

Anatomy

It is located on the dorsal surface of the brainstem with the midpoint at the level of the area postrema (AP). The NTS extends caudally from the calamus scriptorius and rostrally from obex. There are three subdivisions; rostral, intermediate and caudal (to the calamus scriptorius). Vagal and glossopharyngeal afferents are contained within a single tract running parallel to the NTS, the solitary tract. Vagal afferents terminate in the caudal two thirds of the nucleus whereas the glossopharyngeal afferents terminate in the rostral two thirds (Cottle, 1964). This data implies a large overlapping area in the intermediate zone of the NTS, a site of cardiovascular integration. More detailed mapping studies of the NTS have ensued and will be discussed below.

Cells contained within the NTS have been shown to have a low degree of convergence with respect to input. One study stimulated the carotid sinus, aortic and vagal nerves and found that ~85% of cells recorded responded from only one input (Donoghue *et al.*, 1985). This data suggests that inputs use distinct, separate neuronal 'channels' for each input type. A further study in the brainstem slice found that just

over 60% of cells within the NTS received only one input form the solitary tract (McDougall *et al.*, 2009). Input to the NTS has been shown to be highly reliable operating in multiple redundancies. For example, upon entering the NTS one pulmonary afferent branches and forms approximately 40 varicosities around a single NTS cell (Anders *et al.*, 1993, Kubin *et al.*, 2006). Electrophysiological studies have estimated that one EPSC recorded in a second-order NTS cell is a result of an average of 20 release sites (Andresen & Peters, 2008, McDougall & Andresen, 2013). This organization makes the primary afferent input extremely reliable. The release probability at these synapses is extremely high when compared to others in the brain (Doyle & Andresen, 2001).

Subsequent to the afferent primary termination in the NTS onto second-order cells a variety of projections are possible (see figure 1.5). Projection to higher order neurons can occur before exiting the NTS allowing further processing of inputs. There is also the presence of interneurons adding another layer of complexity to these connections and are both excitatory and inhibitory (Izzo *et al.*, 1992). It should also be noted that there is a diverse array of neurotransmitters acting to modulate the excitatory and inhibitory cells within the NTS and will be discussed below. The NTS also receives multiple inputs from central areas (see figure 1.6).

Figure 1.5 Neuronal circuitry of the NTS

Schematic diagram showing possible organization of the microcircuitry in the NTS based on *in vivo* and *in vitro* evidence (adapted from Andresen & Paton, 2011). Afferents enter the nucleus *via* the solitary tract and synapses onto second order neurons (A, B, C and D). Excitatory connections are shown in red and inhibitory connections are shown in blue.

A – Receive afferent input and then project directly out of the NTS to other targets.

B – Polysynaptic pathway receiving GABAergic interneurons then projecting to higher order neurons before leaving the NTS.

C – Polysynaptic pathway projecting to higher order neurons before leaving the NTS.

D – GABAergic interneuron

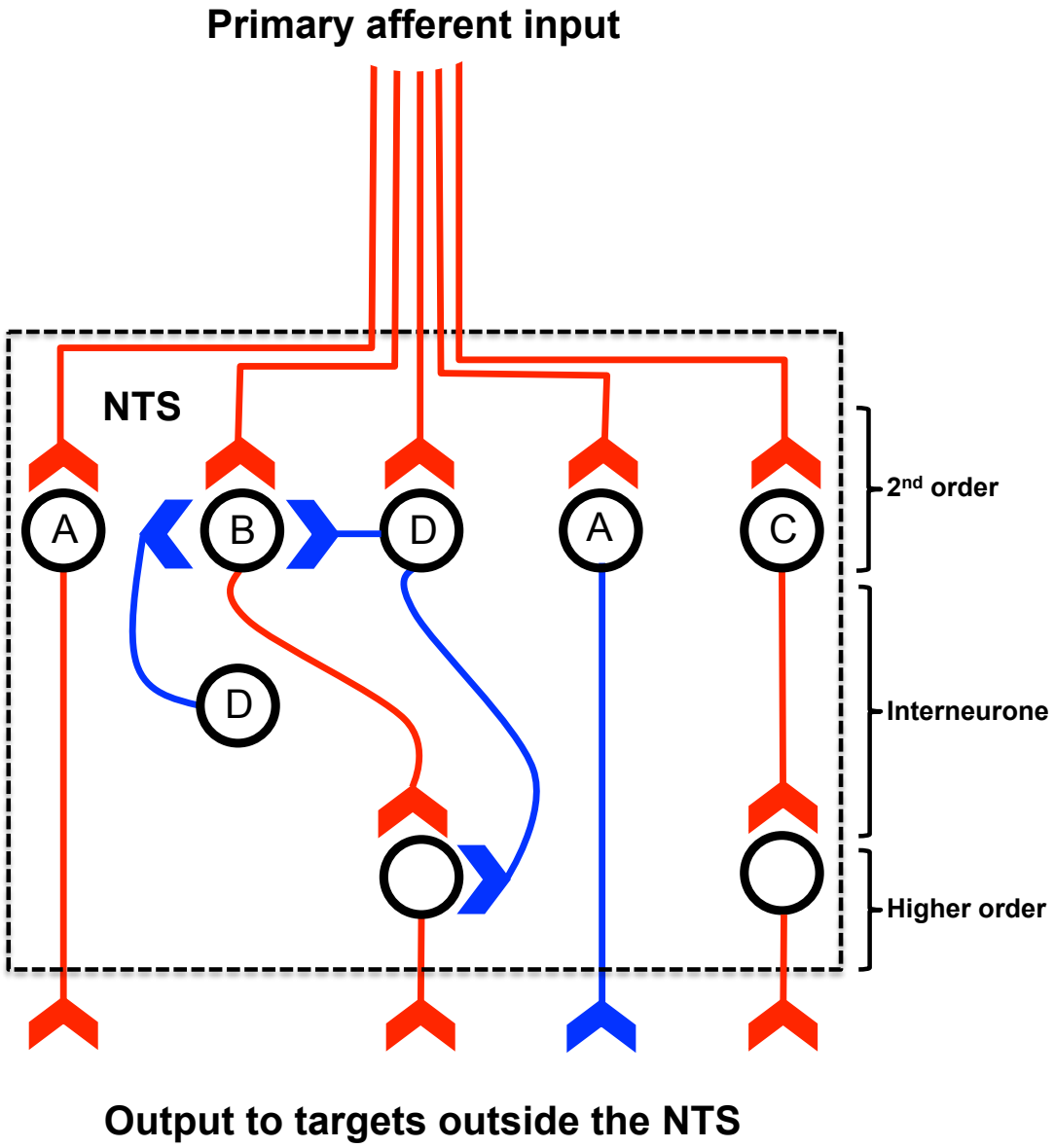

Primary afferent input
NTS
A
B
D
A
C
D
2nd order
Interneurone
Higher order
Output to targets outside the NTS

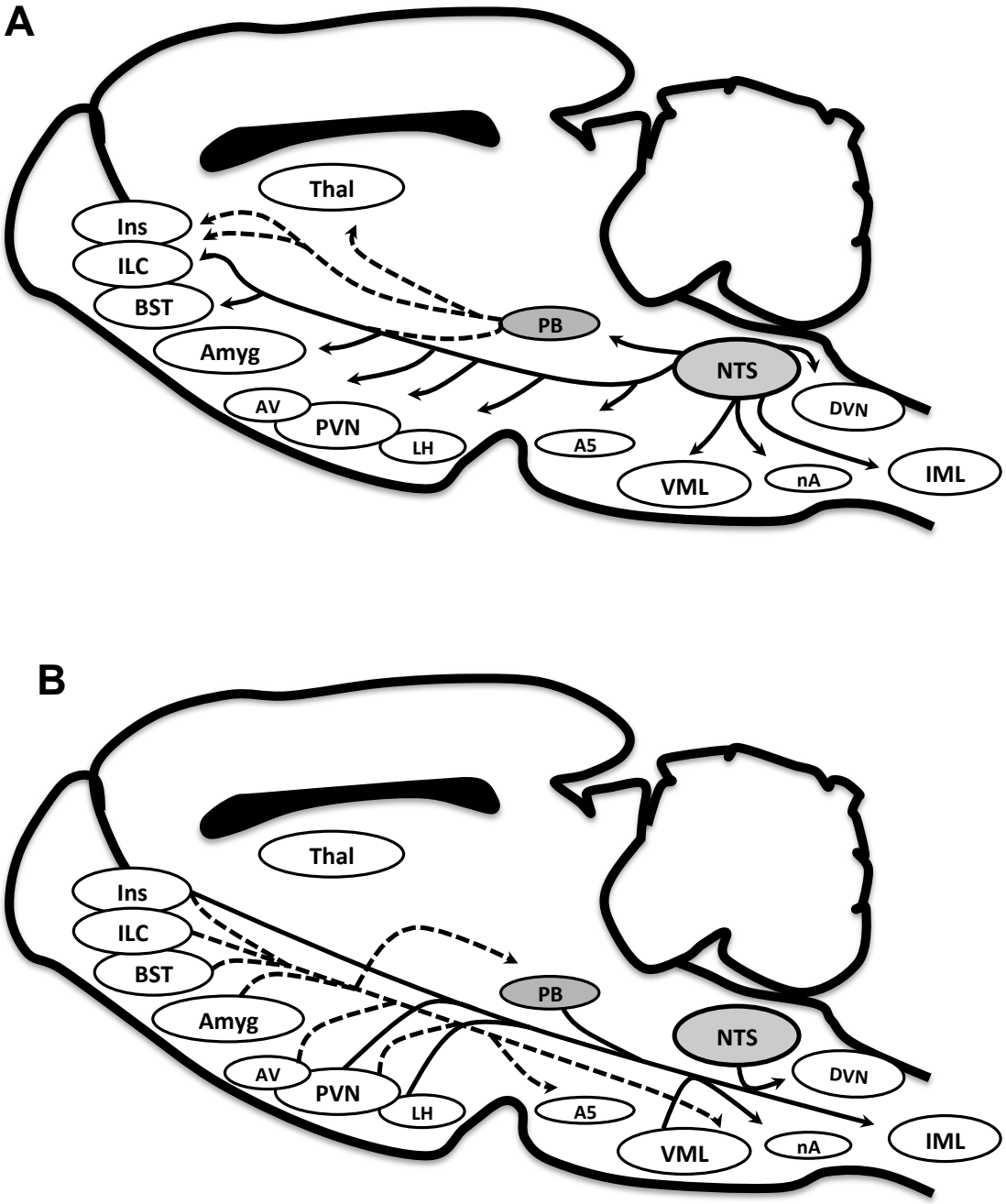
Figure 1.6 Central autonomic afferent and efferent systems

Schematic diagram detailing afferent and effect connections of central cell groups known to be involved in autonomic processing in the rat (adapted from Saper, 2004).

A) Ascending: Cell groups receive direct afferent input from the NTS (solid line) and/or indirect input from the parabrachial nucleus (dashed line).

B) Descending: Direct efferent projections to preganglionic cell groups (grey) in the brainstem (solid line) and/or indirect inputs form premotor areas (dashed line).

A5	A5 noradrenergic group
Amyg	Central nucleus of the amygdala
AV	Anteroventral third ventricular area
BST	Bed nucleus of the stria terminalis
DVN	Dorsal vagal nucleus
ILC	Infralimbic cortex
IML	Intermediolateral cell column
Ins	Insular cortex
LH	Posterior lateral hypothalamus
nA	Nucleus ambiguus
NTS	Nucleus tractus solitarius
PB	Parabrachial nucleus
PVN	Paraventricular nucleus
Thal	Ventroposterior parvocellular nucleus of the thalamus
VLM	Ventrolateral medulla


A
Thal
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DVN
AV
PVN
LH
A5
VML
nA
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B
Thal
Ins
ILC
BST
Amyg
PB
NTS
DVN
AV
PVN
LH
A5
VML
nA
IML

1.6.2. Neurochemistry of the NTS

The neurochemistry of the NTS is one of the most diverse of all brain areas. The literature suggests a role in cardiovascular regulation of a wide range of signalling molecules and the receptors present in the NTS. While an exhaustive list of these systems individually present is not possible, those of interest with respect to the aims for the thesis will be reviewed in more detail.

Glutamate

Glutamate is known to be the major transmitter in the NTS (see Talman, 1997, Baude *et al*., 2009) and its dynamics have been reviewed as part of the previous sections. Interestingly, only 17% of NTS neurons excited by aortic baroreceptor afferent stimulation were inhibited by the NDMA receptor antagonist AP-5 while the non-NMDA antagonist CNQX inhibited 50% (Zhang & Mifflin, 1998). This suggests that there may be other neurotransmitters involved in afferent processing within the NTS.

Υ-Aminobutyric acid (GABA)

The NTS contains a high concentration of GABA (Dietrich *et al*., 1982). Blockade of $GABA_A$ receptors within the NTS were found to facilitate transmission in the NTS evoked by vagal stimulation (Bennett *et al*., 1987). This indicates that vagal stimulation causes GABA release in the NTS. This study and another study by Jordan *et al*. (1988) found that spontaneous activity recorded in NTS neurons was augmented by $GABA_A$ blockade suggesting a tonic release of GABA in the NTS. Interneurons present in the NTS are thought to be the source of the released GABA (Maqbool *et al*., 1991). The same study traced vagal afferent inputs and found them to terminate onto GABA immunoreactive cells. Evidence predominates for GABA-mediated inhibition to be exclusively at the post-synaptic level and not released from vagal afferent terminals.

Glycine

While microinjection of glycine into the NTS can cause bradycardia and hypotension or hypertension, strychnine causes no effect on baroreflex (Kubo & Kihara, 1987, Talman & Robertson, 1989). Further, strychnine had no effect on vagal afferent-evoked inhibition of NTS neurons (Wang *et al*., 2010). Together this data suggests that while glycine receptors are present in the NTS, there is no evidence for glycine being released by either reflex activation or vagal stimulation.

Acetylcholine

Acetylcholine is indeed present in the NTS (Helke *et al*., 1980) and cells within the NTS express choline acetyltransferase, the enzyme required for acetylcholine systhesis (Helke *et al*., 1983). One *in vitro* study found activation of glycine receptors caused release of tritiated acetylcholine loaded into cells by addition to the incubation medium (Talman *et al*., 1994). Microinjection of acetylcholine into the NTS cause hypotension and bradycardia, which was inhibited by atropine (Criscione *et al*., 1983). Atropine alone caused a small increase in blood pressure and attenuated the baroreflex. Microinjection of physostigmine, an acetylcholinesterase inhibitor, alone caused a mild hypotension and bradycardia. Together, this data suggests that acetylcholine present within the NTS, is tonically released and also released during the activation of the baroreflex.

Nitric Oxide

Nitric oxide synthase, the enzyme responsible for synthesizing nitric oxide (NO), is present within the NTS (Vincent & Kimura, 1992). Experiments microinjecting NO donors into the NTS produced varied effects on blood pressure and heart rate in anesthetised rats (see Krukoff, 1999). Blockade of NO production by L-NAME microinjection into the NTS caused a decrease in heart rate and blood pressure, suggesting NO is tonically released (Matsumura *et al*., 1998). Paton *et al*. (2001) proposed that NO produced by nitric oxide synthase in the vascular endothelium

inhibits the baroreflex. NO released form the vasculature then diffuses to GABAergic interneurons to modulate their activity.

Adenosine

The NTS has a particularly high density of adenosine uptake sites and the adenosine deaminase, which is responsible for adenosine turnover in tissue (Bisserbe *et al*., 1985, Lawrence *et al*., 1998). Caffeine, a non-selective adenosine receptor antagonist inhibit the bradycardia associated with baroreflex activation (Mosqueda-Garcia *et al*., 1989). Selective blockade of adenosine A_1 receptors within the NTS causes an attenuation of the hypertension associated with stimulation of the hypothalamic defence area (HDA; St Lambert *et al*., 1996). It was later suggested that, for the most part, the source of the extracellular adenosine was from metabolism of ATP (St Lambert *et al*., 1997). More recently using enzyme encapsulated microelectrodes adenosine release was directly detected in the NTS as a result of HDA stimulation (Dale *et al*., 2002). Hypoxia was also found to increase adenosine release in the NTS, but only during the period of re-oxygenation of the tissue following termination of the hypoxic stimulus (Gourine *et al*., 2002).

Adenosine triphosphate

The density of the adenosine triphosphate (ATP) receptor P2X is especially high within the NTS (Yao *et al*., 2000, Yao *et al*., 2001). Microinjection of ATP into the NTS produces hypotension and bradycardia anesthetised in rats, which was blocked by the non-selective P2 receptor antagonist suramin (Ergene *et al*., 1994). The same antagonist attenuated the bradycardic response to chemoreflex activation when microinjected into the NTS of the working heart brainstem preparation (Paton *et al*., 2002). However, a more recent study showed that the selective P2X receptor antagonist PPADS has not effect on the hemodynamic changes elicited by activation of the chemoreflex in awake rats (Braga *et al*., 2007). Using enzyme encapsulated microelectrodes Gourine *et al*. (2008) demonstrated that ATP was released in the NTS. This release was postulated to arise from slowly adapting pulmonary stretch

receptors that terminate in the NTS. This ATP release activated second-order relay neurons mediate the Hering-Breuer reflex, which maintains appropriate lung inflation. Later, using optogenetic activation of astrocytes the source of the ATP shown to glial (Gourine *et al*., 2010).

Noradrenaline

Noradrenaline innervation in the NTS arises externally from the locus coeruleus (McBride & Sutin, 1976) and internally from the A2 group of noradrenaline-containing cells (Levitt & Moore, 1979). Blockade of α_2 adrenoceptor by yohimbine and idazoxan inhibited the depressor and bradycardic responses to aortic depressor nerve stimulation (Sved *et al.*, 1992). Yohimbine and idazoxan alone were found to increase blood pressure. This indicates that noradrenaline is release in the NTS both tonically and as a result of baroreceptor activation. Additionally, one study found that noradrenaline efflux was increased in the area of the rabbit NTS as a result of baroreceptor activation (Yamazaki & Ninomiya, 1993).

Dopamine

There is evidence of a population of dopamine neurons in the A2 region of the NTS (Armstrong *et al*., 1982, Gonzalez-Cuello *et al*., 2010). Microinjection of dopamine itself into the NTS causes bradycardia and hypotension (Zandberg *et al*., 1979). D_2 receptors have been found to be present in the NTS but not D_1 (Qian *et al*., 1997). Dopamine has been shown to inhibit inward currents produced in NTS cells in response solitary tract stimulation and was attributed to D_2 receptor activation (Kline *et al*., 2002). However, the D2 receptor antagonist sulpiride had no effect on cardiovascular reflexes when administered centrally (Bogle *et al*., 1990, Dando, 1995). Dopamine release in the NTS has been detected with microdialysis, but only during severe hypoxia (Goiny *et al*., 1991).

5-HT

The role of 5-HT and 5-HT receptors have be discussed in detail in sections 1.4

Peptides

The NTS contains a wide complement of neuropeptides and many elicit cardiovascular effects when applied to the NTS. Substance P, neuropeptide Y, angiotensin II, vasopressin, cholecystokinin, bradykinin, somatostatin, opioid peptides, endothelin and neurotensin have all be shown to be present (see Lawrence & Jarrott, 1996). Substance P is especially of interest in the NTS due to the fact it is often found co-localised in terminals along with 5-HT (Thor & Helke, 1987). Indeed, 5-HT and Substance P may even be co-released (see Hodges & Richerson, 2008).

1.6.3. Vagus nerve

The tenth cranial nerve is the longest in the body and the name is derived from the Latin *Vulgivagus*; literally wandering or itinerant. As the name suggests the vagus is extensively distributed around the body. Despite being better known for being the principle parasympathetic output for the autonomic nervous system it is mainly a sensory nerve. Indeed, 30,000 fibres present in the feline vagus nerve 80% afferent fibres and only 20% efferent (Foley & DuBois, 1937). These afferent fibres a responsible for conveying a wide range of sensory information from various organs in the body including respiratory system, heart, vasculature, gut, kidneys and liver. Primary vagal afferents all pass through the nodose ganglion before entering the brain by way of the jugular foramen and afferents terminate in the brainstem (Agostoni *et al.*, 1957).

Within the brainstem vagal afferents terminate within the NTS, or close by, which is close to the dorsal surface (Kalia & Mesulam, 1980). The nucleus is responsible for integrating afferent information and orchestrating cardiovascular reflexes. Input from vagal afferents is either transmitted to higher centres, local interneurons or directly to

motor nuclei within the brainstem (see figure 1.5). Output *via* activation of vagal preganglionic occurs either directly or after central integration.

1.6.4. Cardiovascular Reflexes

Baroreflex

Baroreceptors are stretch-activated receptors located in the aortic arch and carotid sinus. They monitor blood pressure in order to modulate sympathetic and parasympathetic output to counteract excursions from tight physiological ranges maintaining correct tissue perfusion. This reflex is known to be disrupted in cardiovascular disease (Mortara *et al.*, 1997).

Baroreceptor afferents display a tonic discharge and changes in blood pressure modulate this activity. Hypertension increases baroreceptor discharge whereas hypotension causes a decrease. Increased baroreceptor discharge activates cardiac vagal neurons slowing heart rate and cause sympathetic withdrawal. Conversely, when discharge slows sympathetic drive increases causing vasoconstriction and cardiac vagal tone withdrawal (Spyer, 1990).

Experimentally this reflex can be activated by introduction of vasoactive compounds into the circulation to change blood pressure. Sodium nitroprusside and phenylephrine cause causing vasodilation and vasoconstriction, respectively, are commonly used to activate the baroreflex. It is also possible to activate the reflex mechanically by occlusion of the abdominal aorta or carotid arteries. Alternatively, electrical stimulation of the aortic nerve can be used to activate the baroreflex (Sapru *et al.*, 1981). This method removes the confounding factor of possible changing central perfusion seen with the others described.

Baroreceptor afferents are carried by the aortic depressor nerve and carotid sinus nerve terminating in the NTS (Ciriello *et al.*, 1981). Mapping of carotid sinus baroreceptor with cell bodies in the petrosal ganglion show myelinated and

myelinated projections to the lateral NTS rostral to obex on the ipsilateral side (Donoghue *et al.*, 1984). After integration in the NTS the baroreflex sends an excitatory projection to the nucleus ambiguous that modulates vagal tone. The CVLM also receives an excitatory input form the NTS in this pathway that then activates an inhibitory input to the RVLM to modulate sympathetic tone (see Dampney, 1994).

Chemoreflex

Recently there has been a renewed research interest in the chemoreflex due to the rise in obstructive sleep apnoea. This condition results in significant blood oxygen desaturation and increases in dissolved carbon dioxide during bouts of apnoea (Sunderram & Androulakis, 2012). These changes in blood gas tensions and pH are all monitored by chemoreceptors located in aortic and carotid body, of which the latter are thought to be responsible for the majority of the chemoreception *in vivo* (Lahiri *et al.*, 1981). Decreases in PO_2, increases in PCO_2 and increases in hydrogen ion concentration all increases in discharge from single fibres from the carotid body or sinus nerve. Decreases in PO_2 cause an exponential increase in fibre discharge whereas PCO_2 is linear (Biscoe *et al.*, 1970, Fitzgerald & Parks, 1971). Interestingly, when both PO_2 and PCO_2 are reduced simultaneously the effect is greater than the sum of each individual stimulus (Lahiri *et al.*, 1981).

Experimental activation of the chemoreflex has achieved by introducing cyanide, inorganic phosphates or CO_2 –saturated saline into the carotid bifurcation. For example *via* the lingual artery with the external carotid artery ligated (Hilton & Marshall, 1982). More commonly, administration of sodium or potassium cyanide in the systemic circulation is used to activate the chemoreflex. The effect of which is abolished by sinus nerve transection (Franchini & Krieger, 1993). Cyanide is thought to cause local tissue hypoxia by temporarily inhibiting cellular respiration and activating the chemoreceptors.

The cardiovascular and respiratory response to activation of the chemoreflex is often variable between species and experimental conditions. In the anaesthetised, freely-breathing rat carotid body stimulation causes hyperventilation, tachycardia and

vasoconstriction (Marshall, 1987). However, when ventilated at a constant rate the tachycardia observed was reversed to bradycardia. It is thought that the hyperventilation caused by the chemoreflex induces a tachycardia and overcomes the bradycardia. Anaesthesia also influences the chemoreflex. So-called 'depressive' anaesthetics such as pentobarbitone have been shown to attenuate the reflex (Marshall, 1987). In awake behaving rats activation of carotid chemoreceptors by sodium cyanide cause profound bradycardia and hypertension (Haibara *et al.*, 1995).

Chemoreceptor afferents project to the NTS *via* the glossopharyngeal nerve with their cell bodies in the petrosal ganglia. When antidromically stimulated these afferents were found to project most often to the commissural subnucleus of the NTS caudal to obex (Donoghue *et al.*, 1984). All projections had a conduction velocity indicative of unmyelinated fibres. Microinjection of kynurenate into the NTS of anesthetised rats abolishes the baroreflex response (Zhang & Mifflin, 1993). Further, microinjection of kynurenate into the RVLM inhibit the sympathoexcitation caused by activation of the chemoreflex (Koshiya *et al.*, 1993). Projections from the NTS to the nucleus ambiguous are thought to mediate the bradycardiac component of the reflex, similar to that seen in the baroreflex.

Cardiopulmonary reflex

This autonomic reflex was first described by von Bezold and Hirt in 1867, and further characterised by Jarisch and Richter, as a pronounced bradycardia, sympathoinhibition and hypotension (see Krayer, 1961). The Bezold-Jarish reflex involves the activation of chemosensitive vagal afferents located in the heart and lungs. It refers specifically to the activation of C-fibre afferents in the coronary circulation (Dawes & Comroe, 1954). Classically, it is activated by introduction of veratum alkaloids into the coronary artery. However, the cardiopulmonary reflex can be activated by stimulation of a range of unmyelinated mechano- and chemo-sensitive cardiopulmonary afferents.

The cardiopulmonary reflex is activated by intra-atrial injection of the 5-HT_3 agonist phenylbiguanide (PBG) which selectively activates peripheral C-fibre terminals as they express 5-HT_3 receptors (Kay & Armstrong, 1990). This elicits the characteristic response of bradycardia, hypotension and apnoea, which is then followed by rapid shallow breathing. Physiologically, mechanical activation of pulmonary afferents when capillary pressure rises, for example during pulmonary oedema where these affects would be beneficial (see Paintal, 1995).

The unmyelinated cardiopulmonary afferents travel *via* the vagus nerve and terminate in the NTS; rostral to the obex in the more medial portions and caudal to the obex is the dorsal commissural subnucleus in rats and rabbits (Gieroba *et al.*, 1995). The authors determined the distribution of c-Fos positive neurons in the NTS after repeated injection of PBG. The reflex is though to be mediated by glutamate release in the NTS as it is completely abolished by bilateral microinjection of the non-selective glutamate antagonist kynurenate (Verberne & Guyenet, 1992). The same study also found that microinjections of kynurenate in the CVLM and bicuculline into the RVLM attenuate the sympathoinhibition associated with the reflex. This suggests that there is an excitatory pathway projecting from the NTS to CVLM and inhibitory pathway from the CVLM to the RVLM mediating the reflex. This is pathway is similar to that seen the in baroreflex. Interestingly, one study in cats found that neurons contained within the medullary raphé were also activated by the cardiopulmonary reflex suggesting additional sites of integration (Vayssettes-Courchay *et al.*, 1997).

1.7. Assessing neurotransmitter release

The detection of neurotransmitter release has mostly been indirect. The use of electrophysiological techniques to measure the effect of receptor activation is by far the most common. However, many other factors affect the electrophysiological response to neurotransmitter release so more direct measurements were sought. Some of the first attempts included the use of 'cortical cups' where a small nylon vessel filled with physiological saline is placed on the cortex and the composition of the saline is determined after removal (Szerb, 1963). This was the first technique that allowed direct assessment of the transmitter molecule itself in response to drug treatment or electrical stimulation. The obvious limitations of this technique being the practical sampling areas and poor temporal resolution as the experimenter has to wait for analyte diffusion into the collection medium. The invention of the push-pull cannula by John Gaddum allowed access to deeper brain structures but still sampled over a broad area and temporal resolution was not significantly improved (Gaddum, 1961). Later, the microdialysis coupled with high-performance liquid chromatography (HPLC) became the accepted technique to directly measure neurotransmitters and this was accompanied by electrochemical techniques; voltammetry, amperometry and enzyme-linked biosensors. The relative merits of current techniques will be discussed carefully.

1.7.1. Microdialysis

Microdialysis recovers small molecules from the extracellular fluid of the brain by circulating a perfusion fluid through a hollow tube with a dialysis membrane covering a small window at the tip. The recovered fluid is then analysed using a range of techniques to determine the concentration of the chosen analyte(s), this is most commonly HPLC (see Ungerstedt, 1991). The main advantage on this technique is it's excellent selectivity (Borland & Michael, 2007). Chromatograms leave little doubt when identifying a peak caused by compounds recovered from a microdialysis probe. Additionally, a wide range of analytes can be sampled depending on how the dialysate is processed. It is possible to recover and determine the concentration of

biogenic amines (Zetterstrom & Ungerstedt, 1984, Kalen *et al.*, 1988a, Kalen *et al.*, 1988b), GABA, glutamate (van der Zeyden *et al.*, 2008), acetylcholine (Kurosawa *et al.*, 1989) and a range of neuropeptides (Wotjak *et al.*, 2008) from the dialysate. The detection limits for these compounds are extremely low and can often be determined simultaneously in one sample. Further, microdialysis probes can be used to deliver drugs locally to the sampling site *via* retrodialysis and multiple probes can be implanted in one experimental animal.

However, there are also significant disadvantages of this technique that must be considered. Firstly, the temporal resolution is often considerably slower than many processes associated with the release, action and re-uptake of neurotransmitters. Many studies sample at a rate of once every several minutes, however sub-minute sampling has been achieved more recently (Rada *et al.*, 2003, Rossell *et al.*, 2003). The diameter of the microdialysis probe is often greater than 250μm with the dialyzing window in the region of 200-500μm. The disadvantage here is twofold; the sampling area is sufficiently large to preclude specificity of particular brain nuclei and there is the possibility of tissue damage. Indeed, assessment of tissue surrounding implanted microdialysis probes found inflammation, gliosis and swollen axons radiating 1.4mm from the probe surface (Clapp-Lilly *et al.*, 1999). Additionally, dopamine release monitored in the vicinity of an acutely implanted dialysis probe was significantly inhibited (Borland *et al.*, 2005). Interestingly, the same study found that the effects of re-uptake inhibitors were artificially enhanced by implantation of the probe.

1.7.2. Voltammetry

Principles

Voltammetric detection involves electrical oxidation of compounds in aqueous solution with an electrode at a positive (anodal) voltage. The anodal potential removes one or more electrons from the compound and thus oxidises it. These electrons are detected by an amplifier connected to the electrode as an increase in anodal current. In fast cyclic voltammetry the active electrode is a carbon fibre and the voltage is driven rapidly positive using a high speed ramp. Different compounds oxidise at different

voltages, i.e. at different positions on the ramp, and this generates a characteristic current transient that facilitates their identification. Following the positive-going ramp, the voltage is then driven negative. This will reverse the oxidation and produce a re-reduction reaction for many compounds. The re-reduction generates a cathodal current transient, which may also be used for identification of the compound. This is only possible for electroactive compounds which include neurotransmitters such as dopamine, noradrenaline and 5-HT (see Robinson *et al.*, 2008).

To turn this phenomenon into an analytical method requires some simple electronics capable of storing the 'background' current profile produce by the voltage scan which is then subtracted form subsequent scans (see figure 1.7). Changes in this subtracted current can then be calibrated against a known concentration of the analyte to give a semi-quantitative determination of concentration. More specific applications of this technique involve more complex voltage scan protocols in order to optimize detection of the chosen analyte. Scan protocols used to collect the data presented in this thesis will be discussed in more detail in METHODS 2.1.5.

Carbon fibre microelectrodes used for voltammetry in biological systems are extremely small; diameters of approximately 7μm and tip lengths of 50-200μm are common. This allows selective sampling from smaller brain nuclei and very low tissue damage. Electrode tracts from a single carbon fibre implantation were found to be undetectable by light microscopy (Peters *et al.*, 2004). Further, the sampling rate is significantly higher than microdialysis. Voltammetric scans can be applied every 100ms or faster (Phillips *et al.*, 2003, Dankoski & Wightman, 2013). With this sample rate its is possible to resolve the dynamics of neurotransmitter release and re-uptake on a more physiologically relevant scale.

Limitations

Selectivity has long been acknowledged as a problem with voltammetry. There are a number of possible interfering electroactive substances in the brain. Uric acid and ascorbic acid are present at high concentrations and can cause spurious electrochemical signals on voltammograms. Additionally, changes in pH and tissue impedance will affect the current change measured by the active electrode. Criteria

for the positive identification of an electrochemical signal has been carefully considered (see Phillips & Wightman, 2003). Four separate criteria should be verified; electrochemical, anatomical, physiological and pharmacological. First, the voltammogram recorded in the experimental system should be consistent with the analyte of interest taken in a controlled environment. Second, there should be sufficient quantities of the analyte of interest in the anatomical location being sampled to account for the signal recorded. Third, there should be electrophysiological data underpinning a physiological role of the analyte at the concentrations detected. Lastly, pharmacological verification by known inhibitors of synthesis, metabolism, and storage of the purported analyte can be used.

Other disadvantages include the inability to detect the basal concentrations. All changes in current are taken over a current background recorded in presence of basal concentrations of the analyte. Further, the range of analytes is limited to electroactive species only. See Table 1.3 for a comparison of the relative advantages and disadvantages between voltammetry and microdialysis.

History

The earliest attempt to use voltammetry to measure a neurotransmitter in the brain was made by Kissinger *et al.* (1973). Here, a carbon paste electrode was introduced into the caudate nucleus and a ramped voltage (0±1V) applied to the surface. The authors were attempting to detect basal extracellular dopamine, however they conceded that ascorbate was the likely source of the electrochemical signal they encountered in the brain. Using the same technique it was possible to resolve increases in electrochemical current caused by 6-hydroxy dopamine microinjected into the vicinity of the electrode (McCreery *et al.*, 1974). Refinement of the technique by the same group quickly progressed to the measurement of both endogenous striatal dopamine release (evoked by electrical stimulation) and 5-HT in the globus pallidus released by pharmacological stimulation using *para*-Chloroamphetamine (Conti *et al.*, 1978). The same group also reported increased electrochemical currents in the hippocampus in response to electrical stimulation of the median raphe, a nucleus known to be rich in 5-HT-containg cell bodies (Marsden *et al.*, 1979). However, the temporal resolution of this approach was limited to one sample per minute and,

therefore, was unable to accurately determine release kinetics. Furthermore, no pharmacological verification of the electrically evoked signal was attempted so it remained unidentified.

Around the same period a group in France employed a variant of voltammetry, differential pulse voltammetry, in an attempt to measure 5-hydroxyindole compounds in brain tissue (Cespuglio *et al.*, 1981a, Cespuglio *et al.*, 1981b). Differential pulse voltammetry applies voltage square waves (not ramps) to the working electrode. The potential is increased over a range where analyte oxidation is expected in a staircase of small potential increments, in this case 50mV pulses from -0.05 to +0.95 V. Changes in current are sampled at the peak and directly after each pulse in the staircase. Current changes between these two points can be used as an index of analyte concentration. This particular approach oxidises ascorbic acid and DOPAC before 5-hydroxyindoles are oxidised therefore removing these oxidation currents masking those of the 5-hydroxyindoles. However, while the authors of this study did manage to reduce the baseline electrochemical signal recorded in the rat striatum with the specific 5-HT depleting agent *para*-chlorophenylalanine they were unable to confirm the identity of the electrochemical signal due to oxidation of the 5-HT metabolite 5-HIAA at the same potential. Again, sample rates were limited to 1 minute intervals.

Into in 1990's, work from a London-based group showed that dopamine-containing terminals in the striatum could be forced to release 5-HT *in vivo* by pre-loading the animal with 5-HTP and recording the release with voltammetry (Stamford *et al.*, 1990). This phenomenon was not explored further but represented an important proof-of-concept that an uncontaminated 5-HT signal could be detected *in vivo*. However, with the increasing use of brain slice preparations less progress refining 5-HT measurement *in vivo* was seen. Brain slice preparations were used in the first reports of voltammetric detection of electrically evoked 5-HT were both pharmacological and electrochemical characterisation of the evoked signal was achieved (O'Connor & Kruk, 1991). This preparation allowed release of 5-HT to be detected from local terminals within the dorsal raphe nucleus, when stimulated electrically. This approach allowed 5-HT autoreceptor function to be probed (O'Connor & Kruk, 1992).

Later, fast cyclic voltammetry was used to study 5-HT release and re-uptake dynamics in the CNS of Drosophila larvae (Borue *et al.*, 2009). However, the first report of 5-HT release and re-uptake in the mammalian (rat) brain that was comprehensively characterised was from Mark Wightman's group (Hashemi *et al.*, 2009). Here, the authors describe 5-HT release in the substantia nigra upon electrical stimulation of tracts emerging from the dorsal raphe nucleus. Great effort has been taken to confirm the identity of the signal was indeed 5-HT according to an accepted criteria (Phillips & Wightman, 2003). This summarises the progression the technique of voltammetry in neuroscience over the last 30 years. At inception voltammetry was one of a number of methods used to sample extracellular concentrations of neurotransmitter, however, through constant refinements to the technique, fast-cyclic voltammetry has emerged as a viable method of assessing 5-HT release and re-uptake dynamics. This is due to the time and spatial resolution afforded by voltammetry that are not possible with techniques currently available for *in vivo* study.

Figure 1.7 Principles of voltammetry

Fast scan-cyclic voltammetry using a carbon fibre microelectrode with dopamine as an example analyte. Dopamine was the first electroactive transmitter to be measured with voltammetry *in vivo*.

A) Reaction scheme for the reduction and oxidation of dopamine on the surface of a carbon fibre microelectrode driven by an applied voltage. Dopamine is oxidized to Dopamine-*o*-quinone by removal of 2 electrons from the hydroxyl groups attached to the benzene ring.

B) Voltage applied to the carbon fibre microelectrode to drive the oxidation and re-reduction of electroactive molecules. This is often referred to as the voltage 'scan'.

C) Background current generated by the voltage scan at the carbon fibre microelectrode. The shaded area shows the difference between currents recorded in the absence and presence of dopamine.

D) Resultant current from subtraction of the background current recorded in the absence of dopamine from that recorded in the presence of dopamine. This is referred to as the 'faradic current' as it is generated by electrons transferred during reduction and oxidation of a particular molecule.

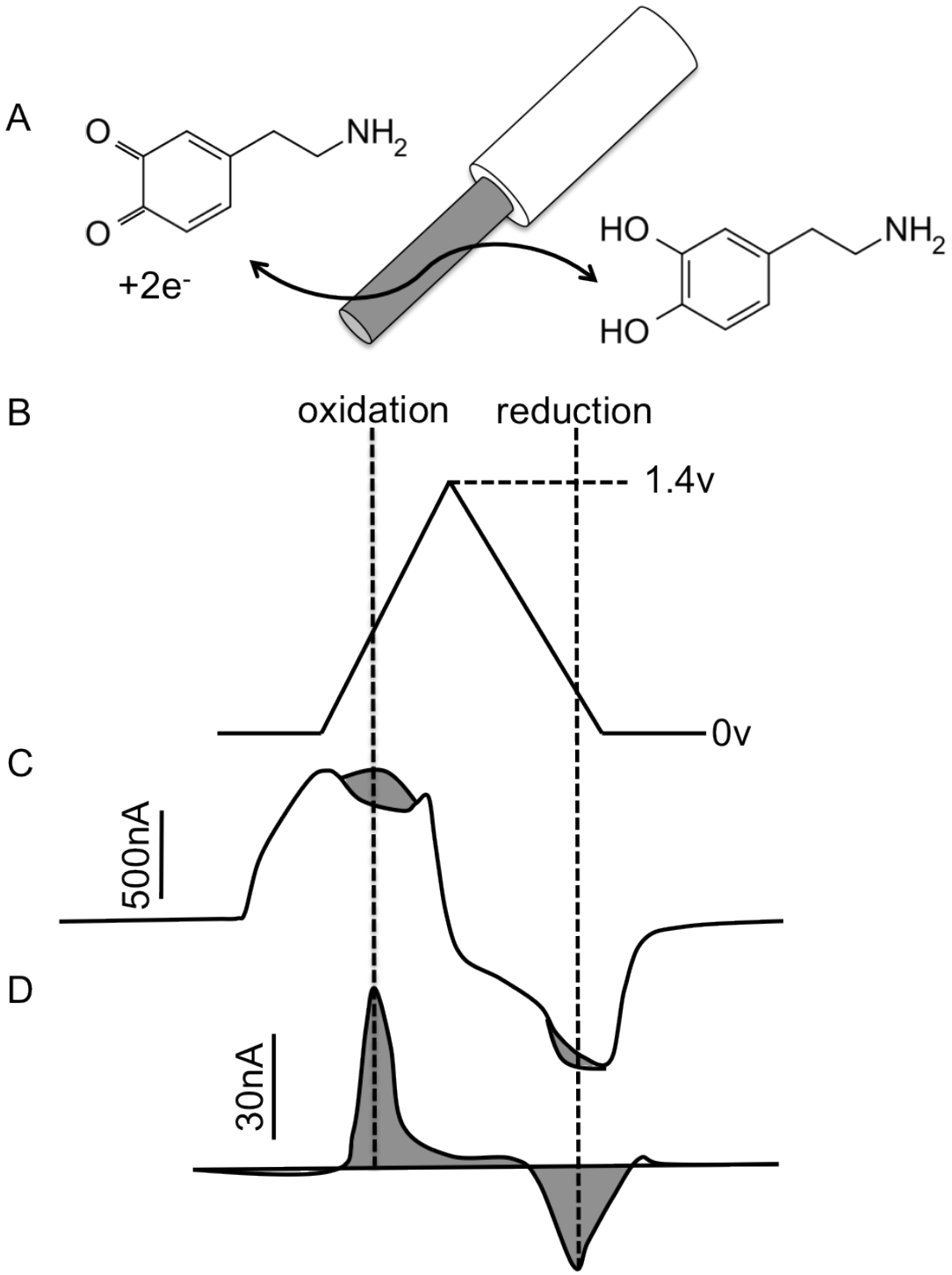

A
O
O
NH2
+2e-
HO
HO
NH2
B
oxidation
reduction
1.4v
0v
C
500nA
D
30nA

Table 1.3 Techniques used to detect neurotransmitters

Comparison of the relative advantages and disadvantages of microdialysis and fast cyclic voltammetry when used to assess the dynamics of neurotransmitter release and re-uptake in biological systems.

	Microdialysis	**Fast Cyclic Voltammetry**
Recording surface dimentions	**~1000-3000μm in length 200-350μm in diameter**	**50-200μm in length 7μm in diameter**
Extracelluar volume sampled	**Large**	**Small**
Sample rate	**Minutes**	**2-10Hz**
Basal concentration detection	**Possible**	**Limited**
Range of analytes	**Wide**	**Limited to electroactive molecules at low potentials**
Selectivity	**Excellent**	**Limited**
Resolve uptake kinetics	**No**	**Yes**
Resolve release kinetics	**No**	**Yes**

1.7.3. Biosensors

Biosensors use a biological component (usually an enzyme) to recognize the analyte. A range of biosensors have been developed for the detection of signaling molecules including ATP, glutamate and lactate (for examples see Gourine *et al.*, 2005, Tian *et al.*, 2009, Brown *et al.*, 2012). These biosensors operate on a similar principle to voltammetry. However, instead of oxidizing the analyte directly, enzymes encapsulated on the surface of the electrode convert the analyte of interest to an electroactive substance (see Dale *et al.*, 2005). One of the enzyme products is usually hydrogen peroxide and easily detectable by applying a constant voltage (usually ~500mV) to the surface of the electrode and the resulting current is monitored. When hydrogen peroxide is oxidized on the surface the current will increase giving an index of local analyte concentration.

The advantage of this system over voltammetry is the ability to detect molecules that are not themselves electroactive. However, an enzyme or an enzyme cascade capable of producing hydrogen peroxide from the analyte is essential. Selectivity is assured by the enzyme recognition being specific for it's particular substrate. Additionally, an enzyme-free electrode identical to the active electrode can be used to detect any non-specific changes in current and can be subtracted from the output of the active electrode. Other advantages include small sensor size (25-50μm in diameter) and fast response time (see Dale *et al.*, 2005).

AIMS OF THIS THESIS

1) Using *in vitro* patch clamp confirm and assess the properties of 5-HT_3 receptors in the NTS and how they exert control on glutamate release. Re-assess the spontaneous 5-HT release first reported in the NTS slice by Wan and Browning (2008). Investigate the effect of re-uptake inhibitors on this spontaneous release of 5-HT and how this affects spontaneous glutamate release. Additionally, assess the effect of non-classical re-uptake inhibitors on this system.

2) Investigate the effects of classical and non-classical re-uptake systems on stimulated release of glutamate in the NTS contained within the brainstem slice.

3) Characterise the electrochemical signal evoked in the NTS during stimulation of the vagus nerve by electrical and physiological means.

4) Assess the efficacy of known re-uptake inhibitors *in vivo* in the nucleus of the solitary tract with microinjections of known concentrations of exogenous biogenic amine. Additionally, assess the properties of non-classical re-uptake systems that may or may not be present in the NTS.

5) Determine and compare the differences between 5-HT release in the NTS under normal physiological conditions and in a rodent model of post-myocardial infarction-induced heart failure. Additionally, glutamate biosensors will be used to assess differences in the dynamics of this transmitter.

2. GENERAL METHODS

The experiments described in the thesis have been conducted both *in vivo* and *in vitro* and the methods have been divided as such for clarity. This methods chapter will start with a general description of the *in vivo* preparation, which is standard for experiments described in Chapters 4, 5 and 6. Specific experiential methods will be described additionally in this chapter or at the start of each method chapter where required, for clarity.

2.1. *In vivo* preparation

All procedures were carried out under license conforming to the Animals (Scientific Procedures) Act 1986. Experiments were performed on male Sprague Dawley rats 180-250g in weight obtained from a colony at University College, London. At the end of each experiment, all animals were humanely killed with an overdose of sodium pentobarbitone (i.v.).

2.1.1. General surgical preparation

Induction of anaesthesia

Animals were placed in an induction chamber and anaesthetised with isoflurane (5% in 100% O_2). The animals were then removed for surgery and anaesthesia was maintained with iosflurane (2-3% in 100% O_2) *via* a rodent facemask (Harvard Apparatus, Kent, U.K). During surgical preparation core body temperature was monitored with a rectal probe and maintained by a thermostatically controlled heating pad (Harvard Apparatus) at 37-38°C.

Vessel cannulation

With animals in a supine position the right femoral artery and vein were cannulated with polyethylene tubing (Portex non-sterile tubing, ED 0.96 mm, ID 0.58 mm). The tubing contained heparinized 0.9% saline (25 UI/ml). The arterial cannula was connected to a pressure transducer (Statham model P23XL) and amplifier (Grass Instruments 7PI) for continuous measurement of blood pressure. The venous cannula was plugged distally until ready for drug administration. All cannulae were secured to the skin, the surgical wound irrigated with saline and the incision was closed with 2-3 sutures.

Tracheal cannulation

The ventral aspect of the neck was exposed with a small midline incision. The salivary glands were separated by blunt dissection and reflected laterally. The trachea was separated from surrounding muscles taking care not to damage the sympathetic nerve trunk and carotid artery. The trachea was cannulated with PTFE tubing (ID 1.5mm) for connection to a small animal ventilator (Harvard Apparatus). Tracheal pressure was monitored via a t-piece connection to a pressure transducer (Statham model P23XL) and amplifier (Grass Instruments 7PI).

Cannulation of right atrium

In some experiments a cannula (Portex non-sterile tubing, OD 0.96 mm, ID 0.58 mm) containing heparinised saline was advanced into the right atrium *via* the right external jugular vein. Until ready for drug administration the tubing was connected to a syringe driven infusion pump for constant rate infusion, described below.

Maintenance of anaesthesia

After successful instrumentation of the animal isoflurane flow was reduced (1-0.5%) and anaesthesia was continued by slow administration α-chloralose (100-120 mg kg^{-1}, i.v.). Isoflurane flow was reduced to zero over a period of 7-10min after α-chloralose administration. Depth of anaesthesia was constantly monitored by paw-pinch and/or

stability of cardiovascular variables. Supplementary doses of α-chloralose (10-20 mg kg^{-1} i.v.) were given if and when required.

Exposure of the brainstem

After cannulation of blood vessels and trachea the animal was placed in a stereotaxic frame (Royal Free Medical Engineering) and secured by ear and an incisor bar clamp. The body was allowed to rest on an adjustable stage and adjusted so the ribcage was clear of the surface. The head was ventroflexed approximately 25° and a midline incision was made from bregma on the skull and continued laterally over the left scapula. The nuchal muscles were reflected laterally by cautery dissection to expose the atlanto-occipital membrane. The membrane was left intact until just prior to electrode placement to reduce exposure of the brainstem and fluid loss.

Exposure of the vagus nerve

The left cervical vagus nerve was exposed blunt dissection from a dorsolateral approach by deflection of the left scapula.
Care was taken when separating the nerves not to damage the others or occlude the carotid artery. When the nerve was free from the bundle it was cleaned of connective tissue, a silk suture was tied distally and the nerve crushed. This prevents any efferent traffic that would directly activate the parasympathetic input to the heart. The nerve was then place on a silver hook bi-polar stimulating electrode. The electrode was connected to a constant current isolated stimulator (Digitimer DS3) triggered by a digital output from a *power*1401+ (CED, Cambridge, UK) controlled by a script written for Spike2 software running on PC.

Correct electrode placement was confirmed by briefly stimulating the vagus (20Hz, 100μA, 1ms pulse width) and observing the resultant bradycardia and hypotension.
The exposed portion of the nerve was completely covered in polyvinylsiloxane dental impression material (Super Dent®, Carlisle Laboratories). This prevented the nerve from drying out and/or fluid build-up causing short-circuit.

2.1.2. Neuromuscular blockade

Once all surgery had been completed neuromuscular blockade was induced by α-bungarotoxin (50 μg per animal i.v.) or decamethonium bromide (1mg kg^{-1} initial, 3mg kg h^{-1}) to maintain stability during recording. The depth and stability of anaesthesia during neuromuscular blockade was assessed by the stability of blood pressure and heart rate and the absence of cardiovascular response to noxious stimuli. An intravenous infusion administered by a syringe-driven infusion pump (6 ml kg^{-1} h^{-1}, Perfusor®, B. Braun) consisting of 50 % Gelofusine plasma substitute and 50 % distilled water containing 100 mM $NaHCO_3$ and 10 mM glucose. Decamethonium bromide was dissolved in the infusion mixture when required. This served to maintain blood volume and prevent metabolic acidosis.

At regular intervals during experiments arterial blood samples were collected and blood gases were monitored using a RAPIDLab 248 blood gas analyser (Siemens Diagnostics, Deerfield, USA. Blood pH was maintained within range 7.35-7.45, *P*CO2; 35-45mmHg and *P*O2; 100-130mmHg. Volume and oxygen content of inspired air were adjusted or injections of sodium bicarbonate 1M (i.v) were made as appropriate to maintain physiological ranges.

2.1.3. Data capture

All recorded variables were digitized to hard-disk drive using a CED 1401Mk2 analogue to digital conversion interface controlled by a PC running Spike2 (Version 6) software (Cambridge Electronic Design). In all experiments blood and tracheal pressures were amplified (Grass, model 7D) and recorded as waveforms. An on-line derivation of heart rate was made from the blood pressure trace using a Spike2 peak count function.

2.1.4. Histology

At the end of each experiment Pontamine sky blue (PSB) dye (1% in saline) was ejected from one barrel of the electrode and/or a dc current of +500nA was applied to the carbon fibre microelectrode *in situ*. The brain was dissected and immersed in 4% formalin saline solution for at least 24 h. The area of interest was then dissected from the rest of the brain and mounted on a freezing microtome (Leitz, Wetzlar, Germany) and when solid cut into 50µm coronal sections. The sections were mounted on gelatin-subbed slides and stained with neutral red. The PSB staining and/or the electrolesion were visualized under a light microscope and recording sites were mapped to a Rat Brain Atlas (Paxinos and Watson, 1998).

2.1.5. Detection of 5-HT

The present study employs adapted fast-cyclic voltammetry (FCV) to measure endogenously released 5-HT and the clearance of exogenously applied 5-HT within the NTS. As previously mentioned, a commonly used technique to measure 5-HT *in vivo* is microdialysis coupled with high performance liquid chromatography (HPLC). While HPLC offers high specificity and the ability to determine basal concentrations of a wide range of neurotransmitters it does not offer the temporal resolution of FCV. This high temporal resolution is required to determine release and reuptake profiles. The general principles have been discussed previously; see INTRODUCTION 1.7.2.

Carbon fibre microelectrodes

Carbon fibre microelectrodes (see figure 2.1) were constructed as follows. Single carbon fibres (7µm diameter) were inserted into 2mm outside diameter glass capillary tubing (Harvard Apparatus Ltd) filled with acetone and cut flush with the end. The acetone was left to evaporate. The filled glass was then loaded into a Flaming Brown

horizontal microelectrode puller (Sutter, Model P80/PC) and pulled to a taper. The fibre was cut with fine dissecting scissors to a length of ~10mm. A small ingot of a low-melting point tin bismuth alloy (Wood's metal) was inserted into the blunt end of the electrode along with a length of copper wire. The alloy was melted with a heat gun to form an electrical contact between the wire and the carbon fibre. The carbon fibre was finished by a process of spark etching using a high voltage DC supply to cut the fibre and etch it to a conical tip approximately 100μm in length (Millar & Pelling, 2001)

'Dident' carbon fibre microelectrodes were used to record endogenously released 5-HT. This electrode incorporates two carbon fibres in a single assembly separated by ~10μm. One of these carbon fibres is used a the active electrode and the other serves as a 'micro reference' that monitors local changes in current unrelated to the voltammetric scan and subtracts them from the final signal. This greatly improves signal to noise ratio (Pennington *et al.*, 2004). They were constructed by bonding two glass capillaries filled with a carbon fibre separated by two further empty glass capillaries with epoxy as shown in figure 2.2. This assembly was pulled to a taper using a Narishige vertical microelectrode puller (Model PE-2) and finished as described above for the single barrelled electrode.

Figure 2.1 Single carbon fibre microelectrode

Schematic representation of a single carbon fibre microelectrode used for *in vivo* voltammetric detection. *Inset* shows the finished tip of the microelectrode after spark etching.

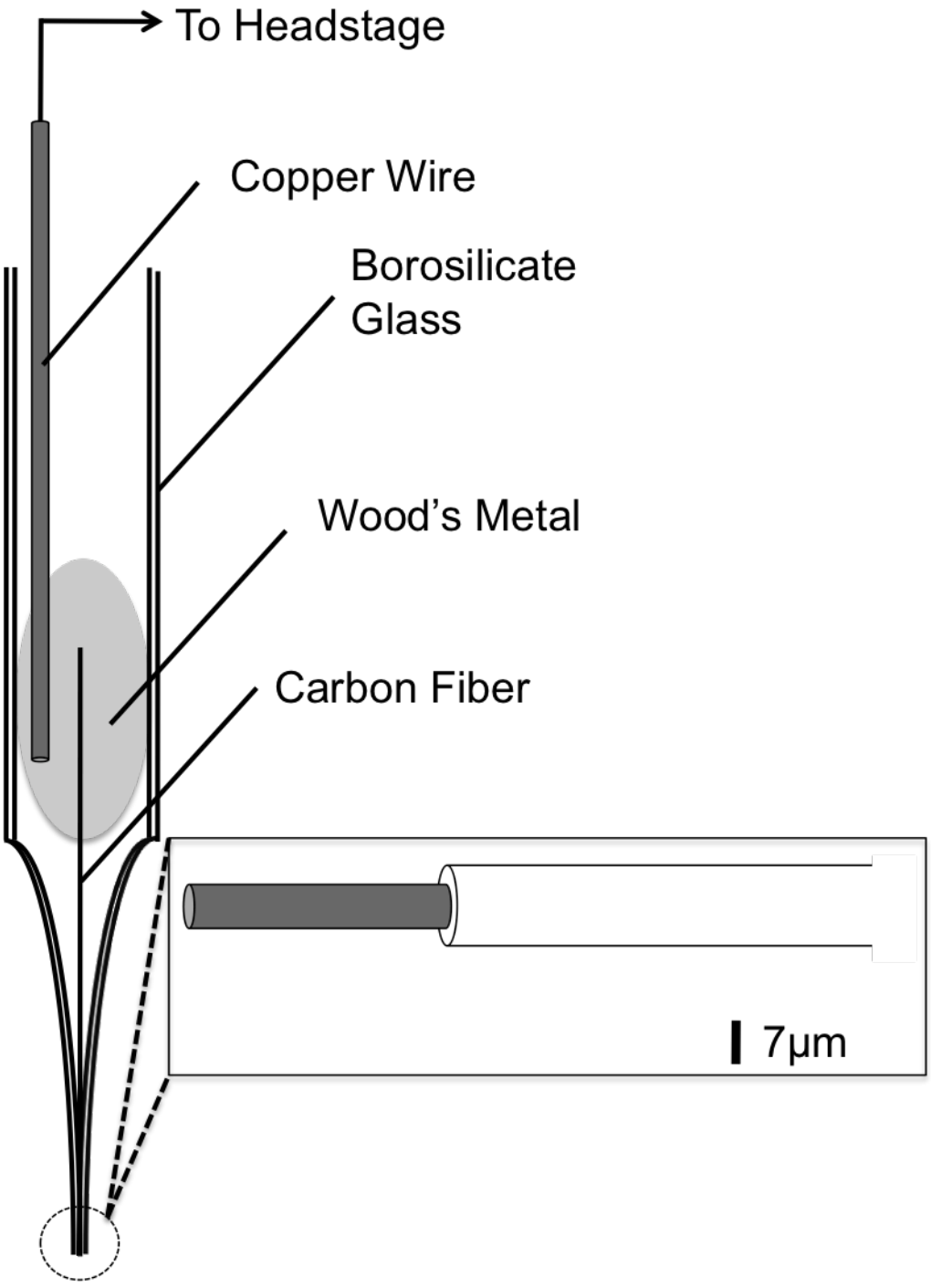
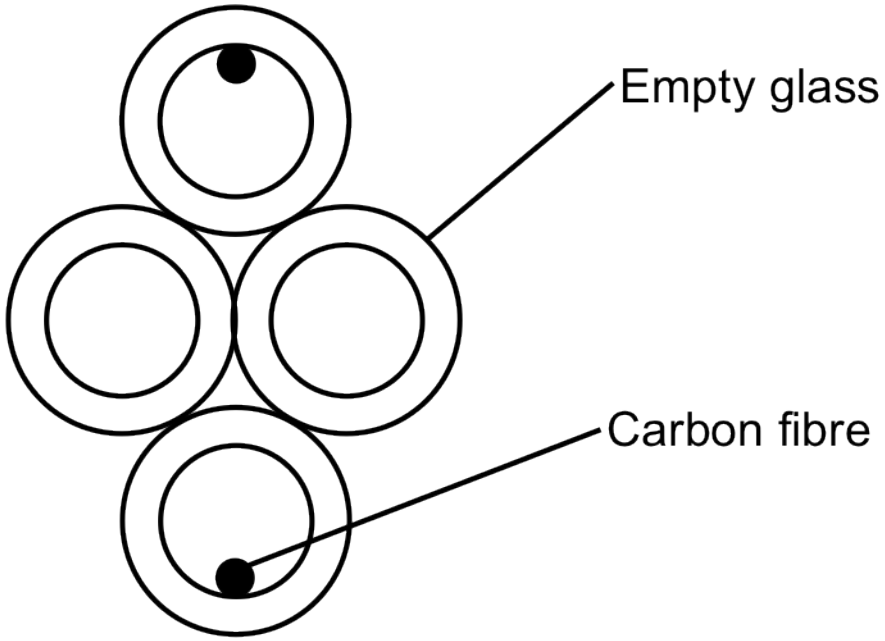

To Headstage
Copper Wire
Borosilicate
Glass
Wood's Metal
Carbon Fiber
7µm

Figure 2.2 Dident carbon fibre microelectrode

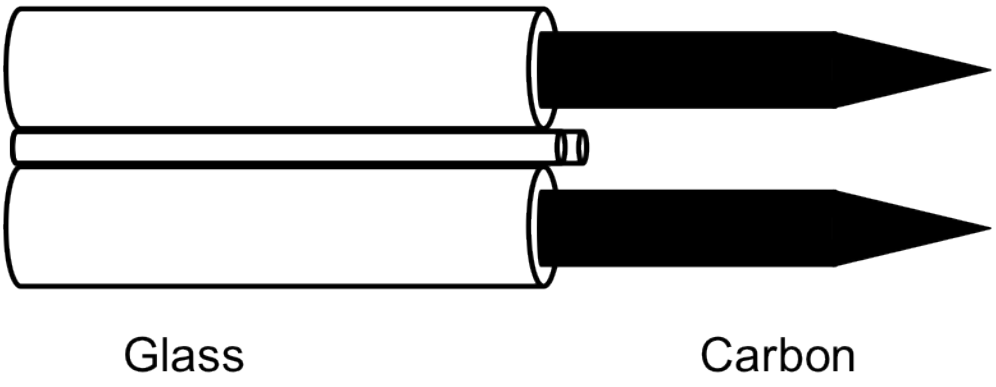
A) Schematic cross-section of an electrode assembly before pulling showing arrangement of empty glass capillaries and carbon-fibre filled glass capillaries.

B) Schematic representation of the tip of a ‘dident’ carbon fibre microelectrode after pulling and finishing with spark etching.

A



B



Reference and ground electrodes

The reference electrode used was a dry-type Ag/AgCl constructed by immersing 1-2mm of silver wire exposed from an insulating tube in bleach overnight. This reference electrode was then positioned on the surface of the brainstem as close to the recording site as possible without disturbing it.
The ground terminal of the voltammetric system was connected to animal *via* the incisor bar of the stereotaxic frame.

5-HT modified Waveform

All voltammetric measurements were conducting using a Millar voltammeter (P.D. Systems, West Mosley, Surrey, UK).

To measure 5-HT *in vivo,* an in-house specially designed trapezoidal ramp waveform was used (see figure 2.3). This waveform improves the selectivity and signal-to-noise ratio for 5-HT detection. Two separate trapezoidal ramps were applied in rapid succession to the carbon fibre electrode at a rate of 2Hz. The first ramp is the 'active' scan as it ramps from 0 to 500mV and then back to -200mV. Using this waveform 5-HT generates an oxidation current peak at ~350mV and a re-reduction peak at ~100mV. The second ramp follows the first after 20ms. This ramp is identical in shape to the first but is offset negatively by 400mV. No 5-HT oxidation (or re-reduction) will occur on this ramp. During these scans, the total current through the electrode has two components, the voltammetric oxidation/reduction peaks plus a 'background current'. The background current is the current that flows simply as a result of the electrode impedance in the solution.

The background current amplitude is normally much greater that the voltammetric currents, so changes in background can produce artifactual signals or noise. For example, changes in tissue impedance *in vivo* due to brain swelling/shrinking can affect the background current. However, the background current depends mainly on dV/dT, so is similar in both ramps. The voltammetric instrumentation electronically superimposes and subtracts the currents during the two ramps (figure 2.4). This process removes most of the background current from the signal leaving the oxidation

and reduction signals intact. During experiments, a background-subtracted signal from a pair of scans was obtained in the tissue. This forms a reference signal. This signal was subtracted on-line from the on-going signal to form the final voltammetric signal (Figure 2.5).

Sampling

Quantification of 5-HT concentration was determined online by sampling the amplitude of both oxidation and re-reduction peaks simultaneously and calculating the differential between the two. This output was then scaled against a calibration taken from a sample of electrodes exposed to a known concentration of 5-HT *in vitro* after use. The carbon fibre electrodes typically lost 50% sensitivity during the course of an experiment. This is seen with other biosensors used *in vivo* (Tian *et al.*, 2009, see Patel & Rice, 2013)

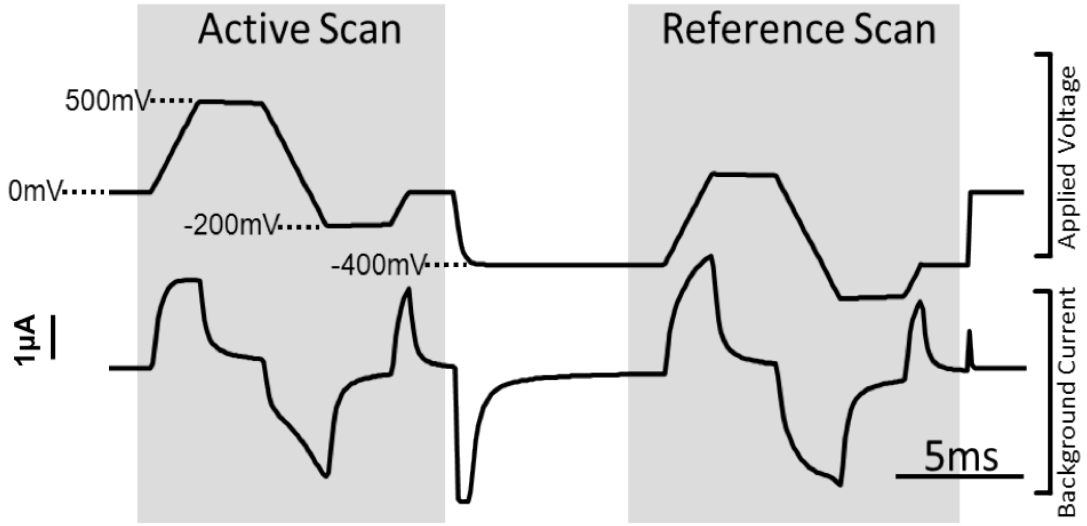


Figure 2.3 Voltammetric waveform

Complete voltammetric scan waveform. Voltage applied to electrode tip and resultant background current. Total scan duration 30ms .

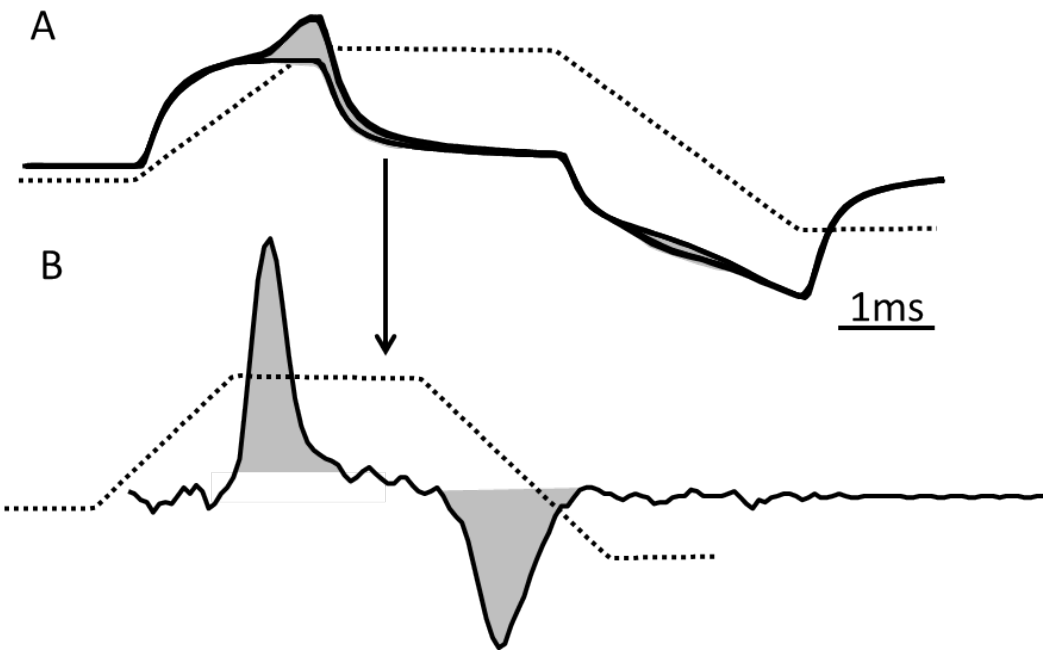


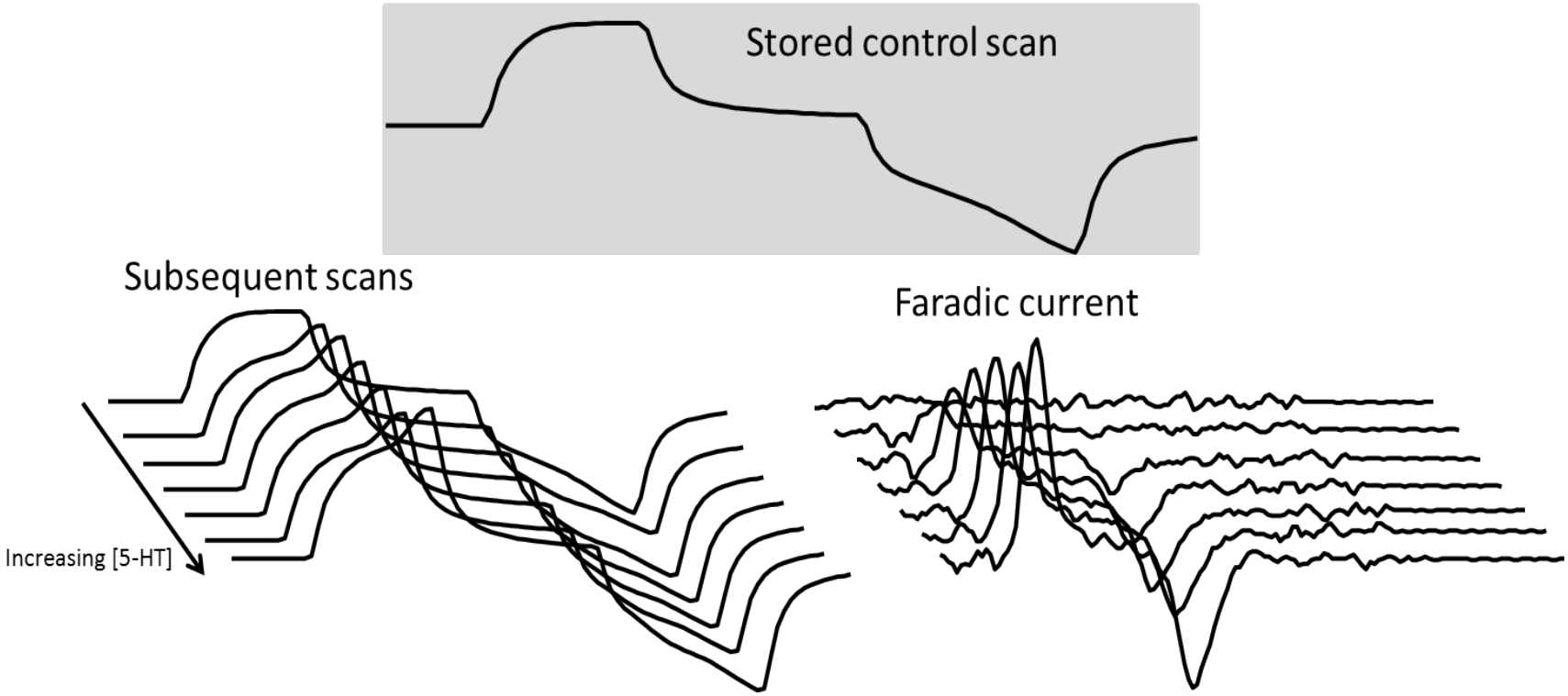
Figure 2.4 Voltammetric signal processing: Stage 1

A) Shows the resultant current from two scans, the shaded area is the increase in faradic current caused by oxidation/reduction of 5-HT.

B) Shows the subtraction of the background current, leaving only the faradaic current.

Figure 2.5 Voltammetric signal processing: Stage 2

Online processing of voltammetric data. Scans were obtained in phosphate buffered saline during increasing 5-HT concentrations. This was achieved by placing an iontophoretic micropipette filled with 5-HT (10mM) alongside the carbon fibre in a recording chamber filled with PBS. Positive current was applied to the 5-HT barrel to eject 5-HT from the micropipette onto the carbon fibre.


Stored control scan
Subsequent scans
Faradic current
Increasing [5-HT]

2.1.6. Exogenous Monoamine clearance

Experimental animals were prepared according to the standard procedure detailed in section 2.1.1.

Electrodes

Electrodes were constructed by modifying a previously described design (Daws & Toney, 2007) A single barrelled carbon fibre electrode prepared and finished with a 100-120µm cone-shaped tip (figure 2.1). Separately, a 3-barrel pipette was pulled and the tip broken back giving each of the barrels an opening of 3-5µm. Under microscopic guidance the carbon fibre microelectrode was positioned 100µm from the tip of the 3-barrel pipette (figure 2.6). The assembly was secured with sealing wax and correct tip-placement was confirmed under the microscope. The shaft of both electrode and pipette were then covered with epoxy resin and allowed to cure overnight.

The 3-barrelled pipette was filled with either 5-HT (100µM), noradrenaline (1mM) or dopamine (1mM) dissolved in phosphate buffered saline (pH7.4), in one barrel and pontamine sky blue dye (15% solution in saline) in another. Ejection of solutions was controlled by pressurising the barrels with nitrogen controlled by a Neurophore (Medical Devices). This was coupled to a timing circuit.

Figure 2.6 Microinjection electrode construction and application

Schematic diagram showing electrode assembly placed within the NTS of the exposed dorsal aspect of brainstem in the anesthetised rat. Voltammetric data was processed online as shown and stored for subsequent analysis.

An enlargement of the electrode assembly tip shows the diffusion of the microinjected 5-HT to the surface of the carbon fibre over a distance of approximately 100μm. It should be noted that the diffusion of the microinjected amines through the extracellular matrix is affected by different factors. These include, but are not limited to, brain region and tissue hypoxia (Rice & Nicholson, 1991, Nicholson & Sykova, 1998).

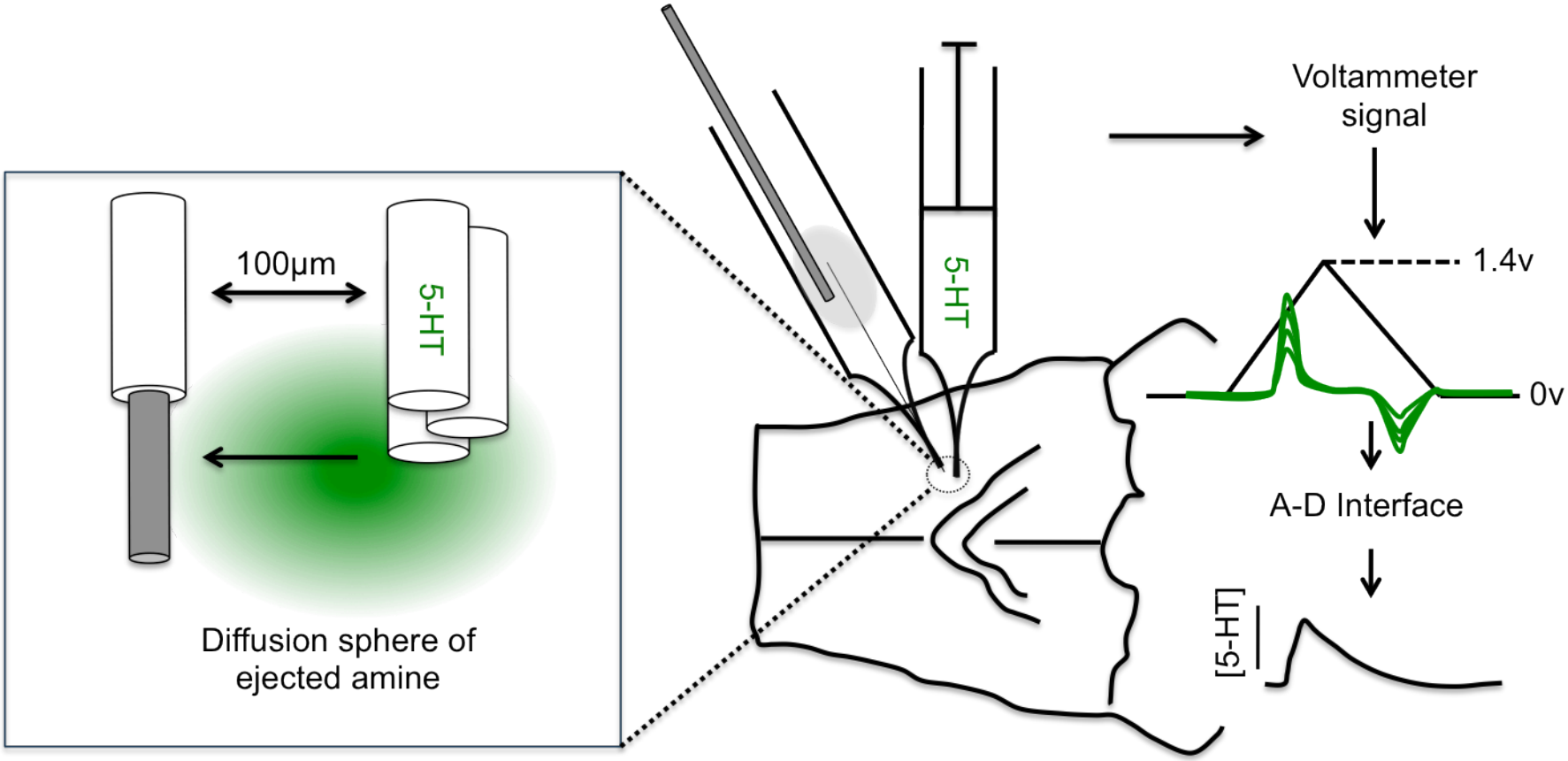
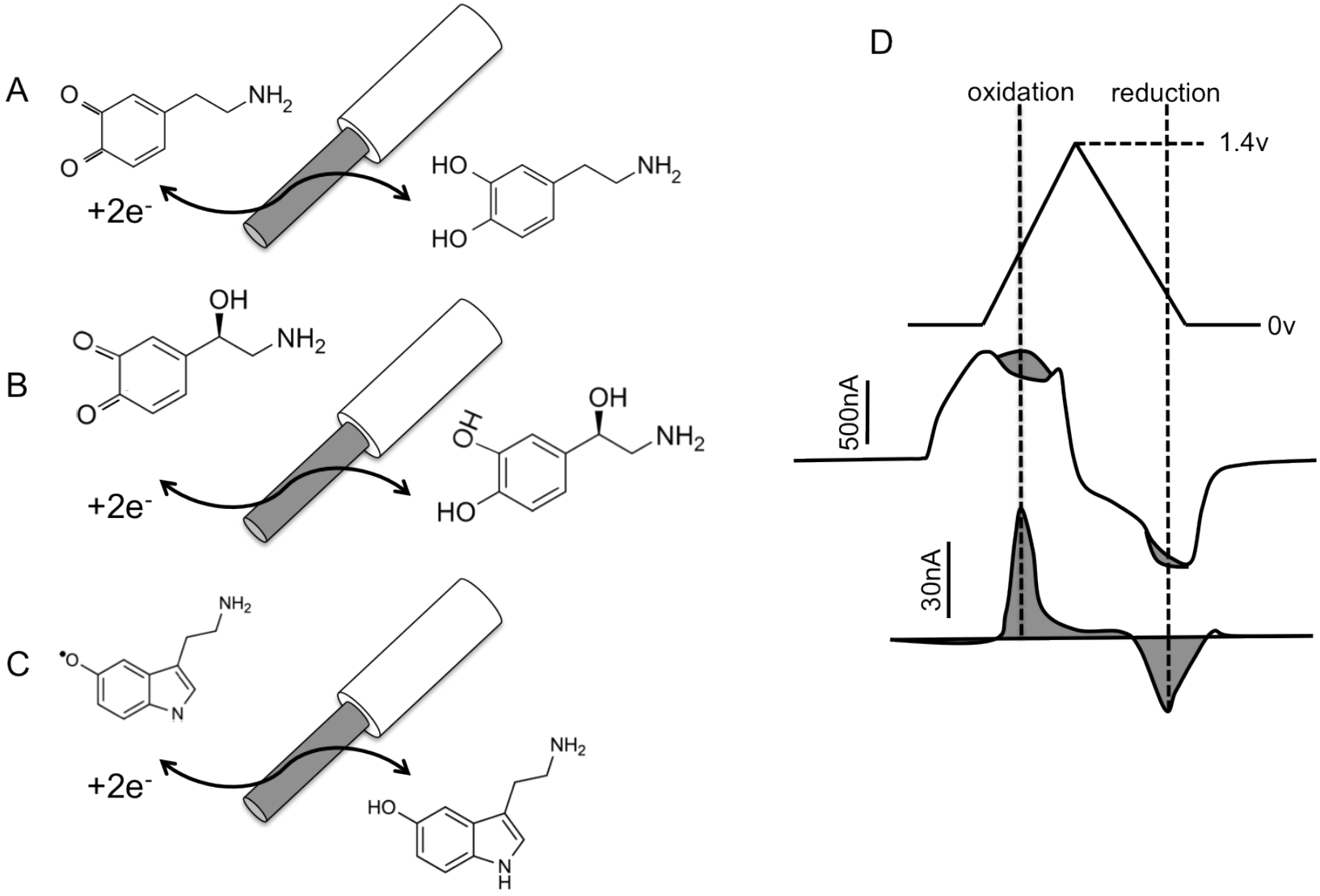

100µm
5-HT
Diffusion sphere of
ejected amine
5-HT
Voltammeter
signal
1.4v
0v
A-D Interface
[5-HT]

Figure 2.7 Voltammetric detection of biogenic amines

Reaction schemes showing the electro oxidation and re-reduction of A) dopamine B) noradrenaline and C) 5-HT using a triangular voltage scan waveform sweeping from 0 to 1.4V. D) Shown the triangular voltage scan waveform and the resulting current when applied to the carbon fibre microelectrode in phosphate buffered saline and in the presence of dopamine.


A
O
O
NH2
+2e-
HO
HO
NH2
B
OH
O
O
NH2
+2e-
OH
OH
HO
NH2
C
NH2
•O
N
+2e-
HO
NH2
N
H
D
oxidation
reduction
1.4v
0v
500nA
30nA

Experimental Protocol

Electrode assembly was lowered into the NTS. The correct position was found using sterotaxic coordinates and recording neuronal activity of the gracile nucleus. When stroking the ipsilateral foot and flank no longer evoked activity then correct positioning was achieved. Additionally, electrode locations were confirmed histologically.

Once electrodes were positioned the voltammetric waveform (Figure 2.7) was applied at a frequency of 8Hz. This voltage waveform allowed measurement of noradrenaline, 5-HT and dopamine but precluded selectively between the three compounds. Background current was allowed to stabilise over a 15-20min period.

Once stable 5-HT, noradrenaline or dopamine microinjections were started. Pressure and time of injection were adjusted so that the peak of the detected monoamine was ~1µM. Monoamine microinjections were applied at 5 min intervals. This timing was found to allow the signal to completely decay before the next application. At least 3 stable applications of 5-HT or noradrenaline were required before administration of test drug or vehicle.

Data Capture and Analysis

The voltammetric signal was recorded continuously over the course of the experiment and derived as previously described to give a relative concentration change. An output from the pressure module was also used to record the time, amount and duration of pressure application. Using both a peak find and decay time algorithm (Spike2, CED) the time taken for the monoamine signal to decay to 80% of its maximum (figure 2.8). This decay time was found to give the maximum differences between control and drug challenges (Daws *et al.*, 1997, Baganz *et al.*, 2008).

Decay time was calculated for three monoamine applications prior to administration of drug or vehicle and averaged. This was used to calculate a percentage change on the decay time of the three subsequent monoamine applications following drug or vehicle challenge (figure 2.9).

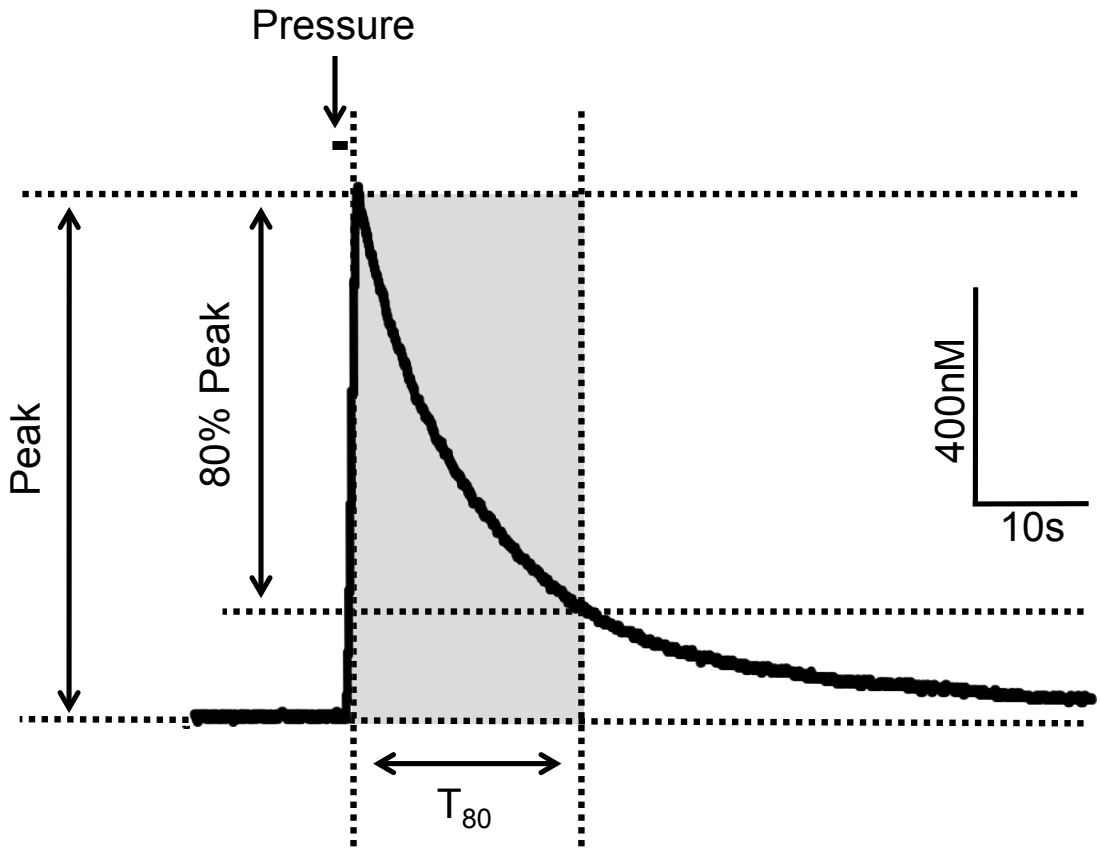


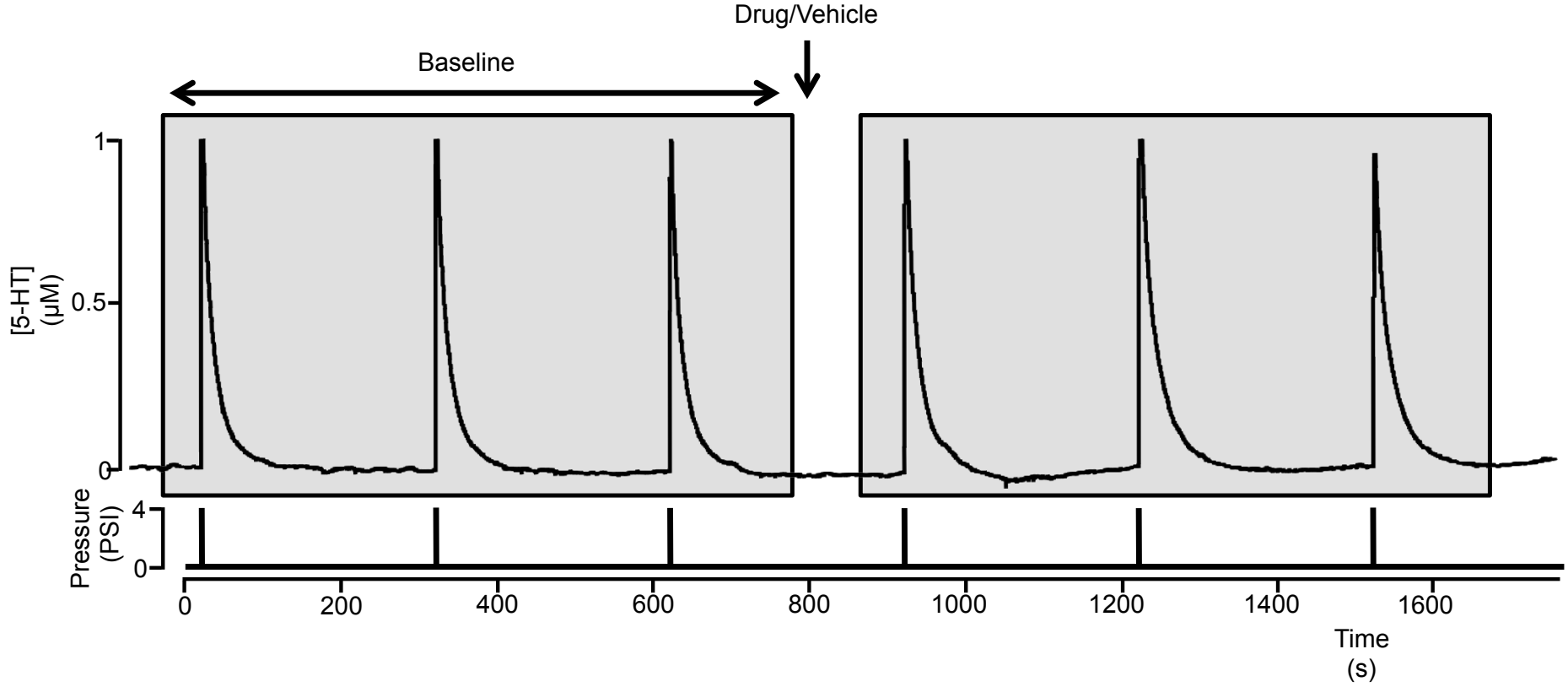
Figure 2.8 Data analysis: Decay time

Sample trace illustrating typical changes in extracellular amine concentration after microinjection of 5-HT (100nM). Analysis was the same for all amines tested.

The maximum concentration change over background was calculated (peak). The decay time (T_{80}) was calculated from the point at which the signal was 80% of the peak value.

Figure 2.9 Data analysis: Quantifying drug effects

Sample trace illustrating a typical experiment. Three microinjections were performed before application of test drug. The T_{80} values for these three microinjections were calculated and averaged as 'baseline'. Drug or vehicle was applied 3 min before the subsequent microinjections. Three microinjections were performed in the presence of the drug or vehicle. T_{80} values for these three microinjections were calculated and averaged. A percentage change compared to baseline was then calculated.


Drug/Vehicle
Baseline
1
0.5
0
[5-HT]
(μM)
Pressure
(PSI)
4
0
0
200
400
600
800
1000
1200
1400
1600
Time
(s)

Statistical Analysis

All data is presented as mean ± standard error of the mean (s.e.m). When comparing three or more treatment groups statistical analysis was performed using a 1-way analysis of variance (ANOVA) to compare drug-treated experimental groups to its time-matched control group. Post-hoc analysis was performed using the least significant difference test (LSD), to calculate significant differences between means of drug and control groups. In some experiments statistical analysis was performed using a 2-way ANOVA where multiple observations needed to be compared. Post-hoc analysis was performed, in this case, using the Bonferroni correction.

When comparing two groups of data statistical analysis was performed using Student's unpaired t-test to compare drug-treated experimental groups to its time-matched control group.

For all statistical analysis, differences between groups were considered significant when $P < 0.05$.

2.2. *In vitro* Patch Clamp Electrophysiology

2.2.1. Brain Slice Preparation

Rat brainstem slices containing the NTS were prepared using methods described previously (Doyle *et al.*, 2004, Wan & Browning, 2008, Zhang *et al.*, 2009)

Rats weighing 75-100g (Charles River, TX) were allowed 1 week acclimatization period before sacrifice. Rats were anesthetised with isoflurane and decapitated. The skull and vertebrae were cleared with a fine rongeur allowing the brain to be rapidly removed and immersed in ice-cold cutting solution (see table 2.1). This process took no longer than 60s. The tissue was left for 3-5min to allow it to completely cool.

The block of tissue was prepared by removing any connective tissue and/or vasculature visible on the surface. Cyanoacrylate adhesive was used to secure the trimmed block (~10mm centered on obex) in the specimen bath of a vibrating microtome (Vibratome VT1200s, Leica microsystems, Germany). The bath was filled with ice-cold cutting solution continually bubbled with 95%O_2/5%CO_2 to maintain a constant pH. Slices were cut at a thickness of 300μm with a sapphire blade (Delaware Diamond Knives, Wilmington, DE, USA). After cutting slices were transferred to a recirculating bath (BSC-PC, Warner instruments, MA, USA) filled with aCSF at room temperature continually bubbled with 95%O_2/5%CO_2 for at least 90min prior to commencement of recordings.

NTS-containing slices were prepared in either coronal plane (figure 2.10) for recording mEPSCs or in the horizontal plane (figure 2.11) when stimulating the tractus. The coronal configuration yielded 2-3 usable slices whereas in the horizontal configuration only a single slice was cut per animal.

Single slices were placed in a perfusion chamber (RC-26GLP, Warner Instruments, CT) and continually perfused with aCSF (see table 2.1) at room temperature (2-3ml min^{-1}). Solutions delivered under gravity from 60mL reservoirs and controlled by a series of 3-way valves. The slice was held in place with a slice anchor which consist of type 316 stainless steel with Lycra® threads, finished with a plastic coating (Warner Instruments, CT, U.S.A.). Neurons were visualized using an upright microscope (Olympus BX50WI) fitted with near-infrared differential interference contrast optics and charge-coupled device camera. The resultant image was displayed on a closed-circuit television. Cells were identified as NTS neurons by location and morphology. In coronal slices recordings were confined to the cordal aspect of intermediate NTS (figure 2.10). In horizontal slices recordings were not confined to a specific NTS region (figure 2.11). Healthy cells (see figure 2.12) were identified by their characteristic spindle or oval shape (Doyle *et al.*, 2004).

2.2.2. Patch pipette fabrication

Patch pipettes were constructed using a horizontal pipette puller (P-2000 Sutter instruments, CA, USA) from thin-walled borosilicate glass with filament (ID 0.90-mm, OD 1.2-mm, GC150F- Harvard Apparatus Ltd, WPI; U.S.A). Electrodes had tip resistances of 3-5MΩ. Immediately prior to recording electrodes were back filled with internal solution with composition detailed in table 2.1.

Table 2.1 Solution compositions for slice electrophysiology

Internal Pipette Solution		
K Gluconate	128mM	
KCl	10mM	
$CaCl_2$	0.3mM	
$MgCl_2$	1mM	
HEPES	10mM	
EGTA	1mM	
ATP	2mM	
GTP	0.25mM	pH adjusted to 7.35 with KOH

Sucrose ACSF Cutting Solution	
KCl	3mM
$MgCl_2$	1mM
$CaCl_2$	2mM
$NaHCO_3$	20mM
$MgSO_4$	2mM
NaH_2PO_4	1.25mM
D-Glucose	10mM
Sucrose	206mM

aCSF	
NaCl	126mM
KCl	3mM
$CaCl_2$	2mM
$NaHCO_3$	20mM
$MgSO_4$	2mM
NaH_2PO_4	1.25mM
D-Glucose	10mM

2.2.3. Cellular recording

Recordings were made of neurons in whole-cell configuration voltage clamped at -60mV using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA). Data was filtered at 2kHz and sampled at 10kHz using p-Clamp8 software (Axon Instruments) and digitized via a Digidata 1320 in or 1440 interface. Data was stored on computer for offline analysis. Neurons were accepted for recording if they had an initial seal resistance greater than 1GΩ and required less than 100pA to maintain the holding potential. Series resistance (access + pipette resistances) during recordings were typically 150-300MΩ.

2.2.4. Electrical Stimulation: Horizontal Slice

All electrical stimulation was conducted in horizontal slices to reduce the contribution of direct activation of interneurons by field stimulation and increase latency from stimulus artifact (Doyle & Andresen, 2001). In all electrical stimulated experiments aCSF contained 25μM gabazine (SR-95531) to block $GABA_A$ –mediated currents. Gabazine was used instead of bicuculline because it has been found to block both glycine receptors and SK-type potassium channels leading to possible confounding of results (Shirasaki *et al.*, 1991, Pedarzani *et al.*, 2001).

Stimuli were delivered by placing a 200-μm-diameter concentric bipolar stimulating electrode (FHC Inc, ME, USA) on the visible solitary tract 1-2mm from the site where recordings were attempted (see figure 2.11). Stimuli were delivered *via* an isolated constant current stimulator (DS4, Digitimer, UK). Pulse trains were programmed and triggered *via* a Master-8 (A.M.P.I, Jerusalem, Israel).

Pairs of stimuli were applied every 20s to evoke EPSCs. Pulse duration (0.1-1ms) and amplitude (10-500mA) was adjusted to produce inward currents of 100±20pA. The stimulus interval was altered to allow the first stimulated current to decay completely before the second stimulus. In a separate set of experiments a 100-pulse train was

delivered to the solitary tract at 20Hz to evoke an increase in spontaneous EPSC frequency post-stimulus. The amplitude was adjusted to evoke maximal EPSC inward currents (100-500μA).

2.2.5. Experimental Protocol and Data Analysis

Coronal Slice: Recording mEPSCs

Frequency and amplitude of mEPSCs were sampled continuously. For the coronal slice experiments these mESPCs were recorded for 10 min in drug free aCSF – control period. To test the whole-cell current recordings were stable the frequency of mESPCs over first 200s was compared with that over the last 200s of this 10 min period. If the percentage difference in frequency between these two values was ≤20% then the recording was considered stable. The mean of these two values was taken as control. This was also applied to the amplitude measurements. The test drug/drugs were then applied and recordings were made over a 10 min window before changing to another drug challenge or to drug free ACSF. The maximum number drug challenges were 3 per cell. Frequency and amplitude of mEPSCs were measured over 200s once the effect of a drug had plateaued (i.e. constant frequency mESPCs over 200s) which was approximately 5-7 min after application. The drug/s were then washed out with aCSF. This was carried out within the 10 min window. In the case phenylbiguanide experiments, the 2nd challenge and higher was given after the recover from the lower doses. In case of the 5-HT experiments all neurons received two challenges of different 5-HT doses. For all experiments with antagonist and combined agonist/antagonist/uptake inhibitor only received on challenge of the antagonist alone or combination.

Horizontal Slice: Recording sEPSCs and Evoked EPSCs

The paired-pulse ratio was calculated as the amplitude of the second current relative to that of the first; alterations in the paired-pulse ratio are suggestive of a presynaptic site of action.

Measurement of increases in EPSC activity due electrical stimulation of solitary tract were always taken 1 min after stimulation had ceased, as this is when the maximum increase in activity was observed and averaged over a 30 s period. Two control stimulations of solitary tract (ST) were taken 10 min apart to determine stability. Drugs were applied 5 min after the 2nd stimulation and the changes in frequency and amplitude were compared to that of the 2nd control stimulation.

2.2.6. Statistical Analysis

All data is presented as mean ± standard error of the mean (s.e.m). When comparing three or more treatment groups statistical analysis was performed using a 1-way analysis of variance (ANOVA) to compare drug-treated experimental groups to its time-matched aCSF control group. Post-hoc analysis was performed using the least significant difference test, to calculate significant differences between means of drug and aCSF control groups.

When comparing two groups of data statistical analysis was performed using Student's unpaired t-test to compare drug-treated experimental groups to its time-matched control group.

For all statistical analysis, differences between groups were considered significant when $P < 0.05$.

Figure 2.10 Anatomy of coronal NTS-containing brain slice

Coronal NTS-containing brain slice as visualised in the recording chamber under low power optics (4x) in preparation of patch-clamp recordings. Overlaid with schematic diagram of anatomical features used to identify recording location (SolIM).

10- dorsal motor nucleus of vagus
AP- area postrema
Sol- Solitary tract
CC- Central canal
Gr- Gracile nucleus
SolIM- Nucleus of the solitary tract, intermediate
SolC- Nucleus of the solitary tract, commissural
SolDM- Nucleus of the solitary tract, dorsonmedial

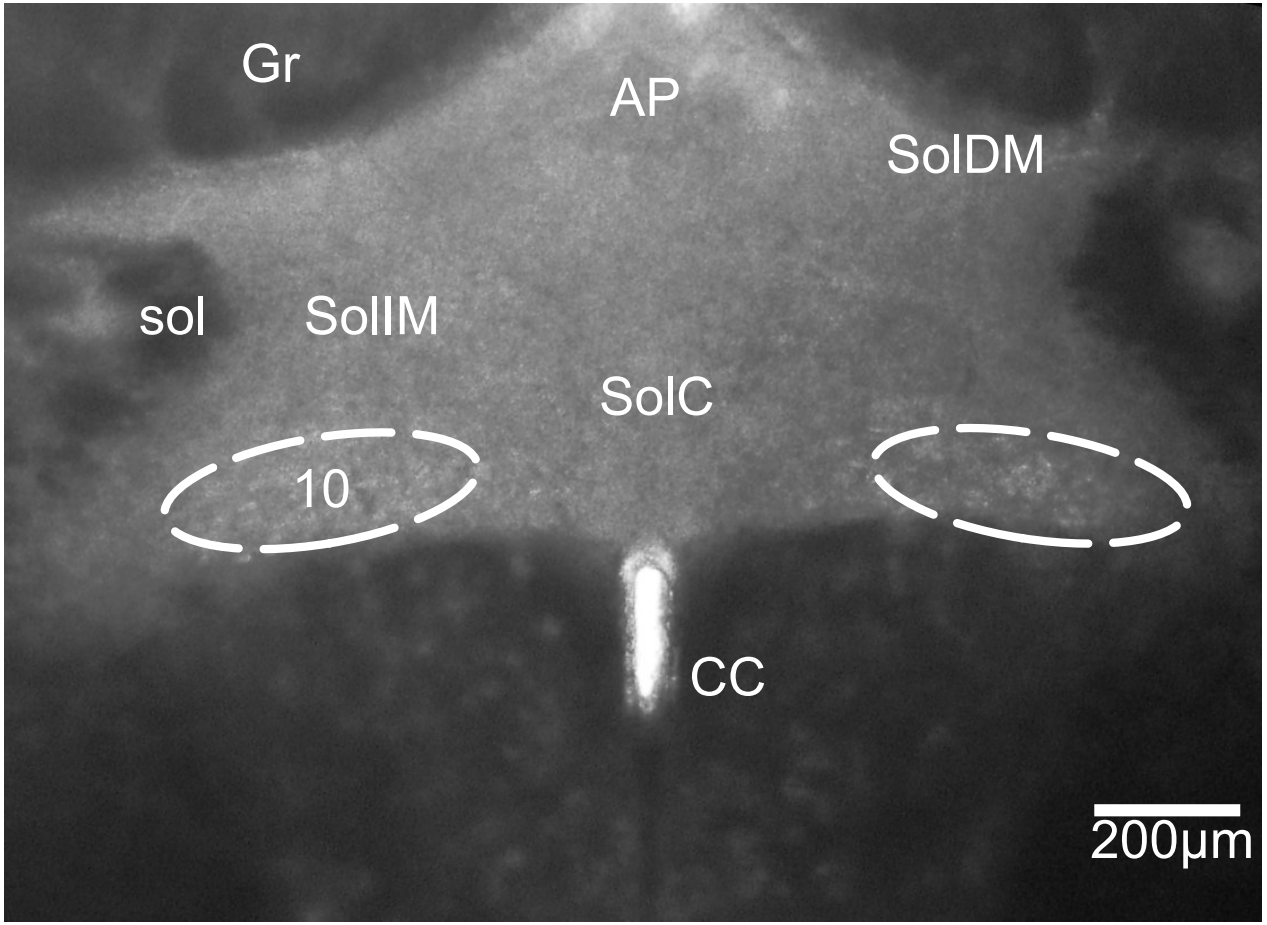

Gr
AP
SolDM
sol
SolIM
SolC
10
CC
200µm

Figure 2.11 Anatomy of horizontal NTS-containing brain slice

Horizontal NTS-containing brain slice as visualised in the recording chamber under low power optics (4x) in preparation of patch-clamp recordings with electrical stimulation of the solitary tract.

sol- Solitary tract
Stim.- Stimulating electrode
4V- 4th Ventricle
NTS- Nucleus of the solitary tract

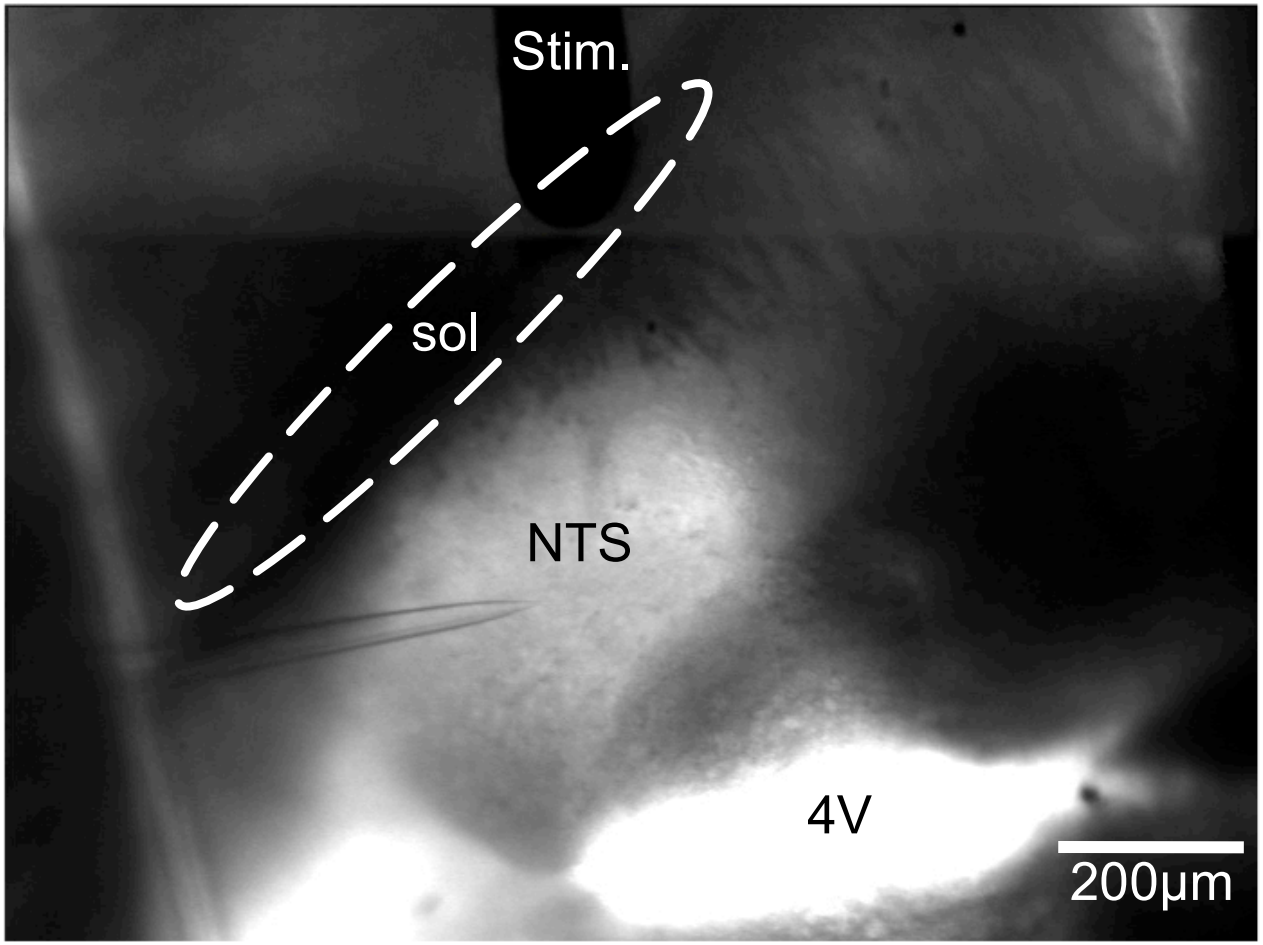
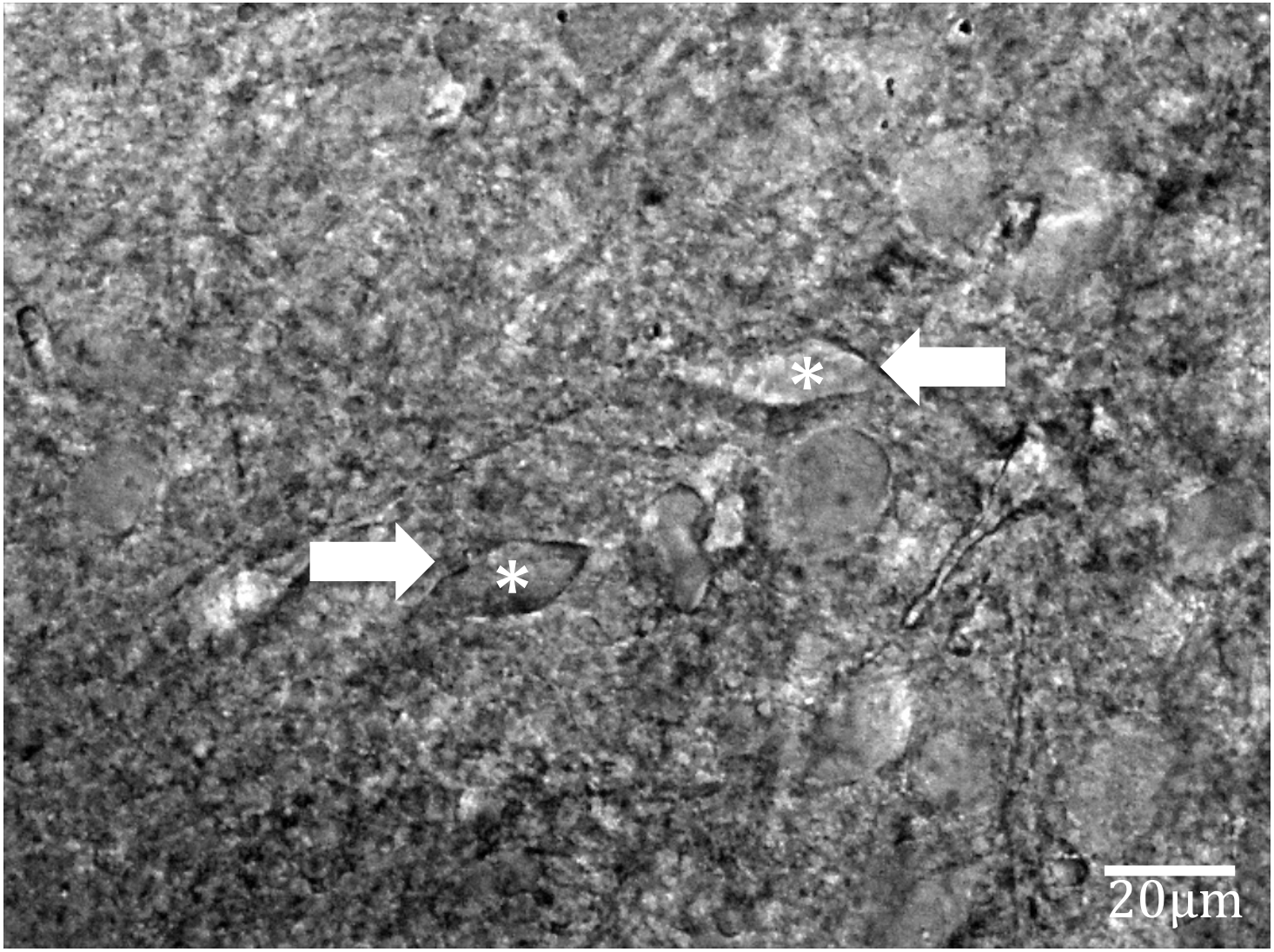

Stim.
sol
NTS
4V
200μm

Figure 2.12 Identification of NTS cells

Photomicrograph taken from a live image as visualised for patch-clamp recordings using differential interference contrast optics. Figure shows the surface of the SolIM region of a coronal NTS-containing slice. Indicated (*) are two cells typical of all those from which recordings were attempted. Note the characteristic size and spindle shape.


*
*
20µm

2.3. Drugs and solutions

The chemical ingredients for aCSF were all obtained from (Sigma-Aldrich, St. Louis, MO, USA)

Dissolved in 0.9 % saline or aCSF:

5-HT creatinine sulphate (Sigma-Aldrich, UK)
5-Hydroxyindole-3-acetic acid (Sigma-Aldrich, UK)
8-OH-DPAT hydrobromide (Sigma-Aldrich, UK)
α-bungarotoxin (Sigma-Aldrich, UK)
α-chloralose (Sigma-Aldrich, UK) was dissolved (10 mg ml^{-1})
Citalopram hydrobromide (AbcamBiochemicals, UK)
Desipramine hydrochloride (Sigma-Aldrich, UK)
DL-p-chlorophenylalanine methyl ester (Sigma-Aldrich, UK)
DL -Fluorocitric acid barium salt (Sigma-Aldrich, St. Louis, MO, USA)
DL -Isocitric acid trisodium salt hydrate (Sigma-Aldrich, St. Louis, MO, USA)
DL -Norepinephrine hydrochloride (Sigma-Aldrich, UK)
Dopamine hydrochloride (Sigma-Aldrich, UK)
Gabazine (Sigma-Aldrich, USA)
GBR-12909 (AbcamBiochemicals, UK)
Granisetron hydrochloride (AbcamBiochemicals, Boston, MA)
Reboxetine mesylate (AbcamBiochemicals, UK)
Phenylbiguanide (Sigma-Aldrich, UK)
Phenylephrine (Sigma-Aldrich, UK)
Pontamine sky blue (BDH, UK)
Sodium cyanide (BDH, UK)
Sodium nitroprusside (Sigma-Aldrich, UK)
Tetrodotoxin (AbcamBiochemicals, Boston, MA)
WAY-100635 maleate, (Sigma-RBI, St. Louis, MO USA)

Dissolved in DMSO:

CNQX (Tocris, USA)
Decynum-22 (Sigma-Aldrich, UK/USA)

Other drugs and reagents:

D-glucose (BDH, UK)
Dimethyl sulfoxide (DMSO) (Sigma, UK)
Gelofusine® (plasma substitute; Braun Medical Ltd, UK)
Isoflurane (Aerrane®; Baxter Healthcare Ltd, UK)
Lignocaine spray (Xylocaine®; AstraZeneca, UK).
Pentobarbitone sodium (Sagatal®; Rhône-Mérieux Ltd, UK)
Sodium bicarbonate (BDH, UK)

3. 5-HT$_3$ AND 5-HT$_{1A}$ RECEPTORS MODULATE SPONTANEOUS AND SOLITARY TRACT EVOKED GLUTAMATE RELEASE IN THE NTS IN VITRO

3.1. Introduction

The 5-HT$_3$ receptor is known to be densely expressed in the NTS, mainly on vagal afferent fibre terminals (Pratt & Bowery, 1989, Reynolds *et al.*, 1989). Following nodose ganglionectomy 5-HT$_3$ ligand binding sites in the NTS were reduced by approximately 50%. This indicated that 5-HT$_3$ receptors are expressed on the vagal afferent terminals themselves. *In vivo* they have been shown to control glutamate release from vagal afferent terminals as blockade of these receptors attenuates vagal afferent excitation (Ramage & Mifflin, 1998, Jeggo *et al.*, 2005). One study using the brainstem slice preparation to study this system within the NTS found that spontaneous glutamate release was attenuated by the 5-HT$_3$ receptor antagonist ondansetron (Wan & Browning, 2008). Further, it was found that this effect was abolished by vagotomy. Taken together these observations suggest that there is a tonic release of 5-HT within the NTS that activates 5-HT$_3$ receptors on vagal afferents increasing glutamate release, at least within the slice preparation. The source of this 5-HT is unknown but it is likely that there is a 5-HT input from raphé cell groups. It is also possible that 5-HT is released from vagal afferents themselves. The current understanding of this system is summarised in figure 3.1.

5-HT neurotransmission is highly regulated by its uptake systems, of which there are several different types (see INTRODUCTION 1.4). The present experiments sought to determine how these different re-uptake systems controlled 5-HT released in the NTS slice preparation. Therefore the present experiments were carried out to investigate the effects of blockade of the 5-HT transporter with citalopram and blockade of the organic cation transporter 3 (OCT3)/plasma membrane monoamine transporter (PMAT) with decynium-22 on mEPSCs recorded in NTS cells within the brainstem slice preparation.

Further, it is widely reported that electrical stimulation of this fibre tract cause inward currents in NTS cells mediated by glutamate release (Doyle & Andresen, 2001, Zhang & Mifflin, 2007). Repeated stimulation of the tractus has also been shown to increase spontaneous activity immediately following the stimulus. This is known as 'asynchronous' release (Andresen *et al.*, 2012). 5-HT_3 antagonists have been shown to attenuate glutamatergic currents with single pulse stimulation (Wan & Browning, 2008). The effect of these reuptake inhibitors on glutamate release elicited by both single pulse stimulation and repeated (train) stimulation of the tractus.

This study was carried out at the Department of Integrative Physiology, University of North Texas (Heath Science Centre), Fort Worth, Texas, USA. The Institutional Animal Care and Use Committee of the University of North Texas Health Science Center approved all experimental protocols.

3.2. Specific aims

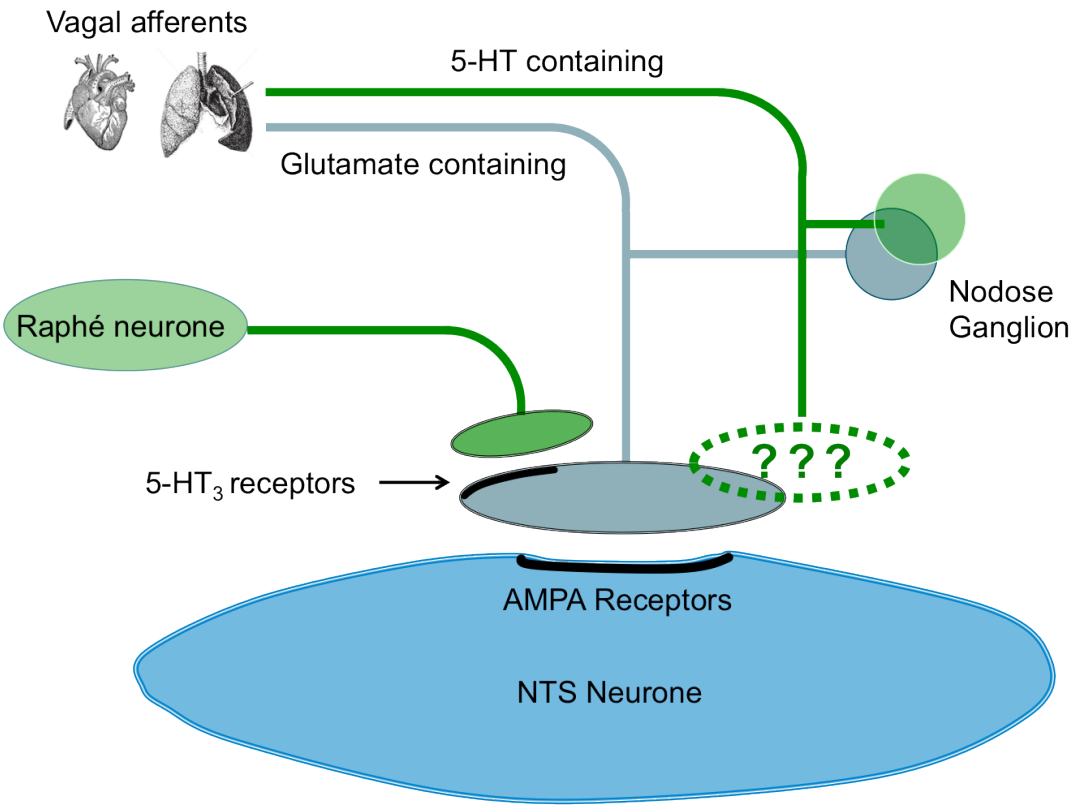
- Reassess the effect of 5-HT_3 ligands on mEPSCs recorded within the NTS and confirm the presence of tonic 5-HT_3 receptor activation.

- Investigate the effect of re-uptake inhibitors citalopram and decynium-22 on this tonically released 5-HT.

- Investigate the effect of 5-HT re-uptake inhibitors on evoked synaptic currents using electrical stimulation of the solitary tract.

Figure 3.1 NTS neuronal inputs

Schematic representation of neuronal input into the NTS. Vagal afferents containing glutamate terminate onto second-order cells within the NTS. Glutamate is spontaneously released from these afferent terminals and activates AMPA receptors expressed by NTS cells. 5-HT-containing terminals from raphe cell groups as well as 5-HT containing vagal afferents also innervate the NTS. Little is known about 5-HT-containing vagal afferents and where they terminate.



3.3. Electrophysiological properties

Spontaneous mEPSCs were recorded in 72 NTS neurons in the presence of 1μM TTX and 25μM gabazine. Baseline mEPSCs frequency was found to be 2.0 ± 0.6 Hz with mean amplitude 13.5 ± 0.8pA. This activity could be completely blocked by the application CNQX (10μM; n=3; Figure 3.2).

Figure 3.2 Effect of CNQX on mEPSCs

Representative mEPSC traces from an intermediate NTS neuron voltage clamped at -60mV. Each panel represents 5 overlapping traces. Bottom trace is recorded in the presence of AMPA/kinate receptor antagonist CNQX (10uM).

3.4. Effect of 5-HT_3 ligands on spontaneous glutamate release

Effect of 5-HT_3 agonist phenylbiguanide

Bath application of the 5-HT_3 agonist phenylbiguanide (PBG; 1μM, n=7) increased mEPSC frequency by 497 ± 112%. Applications of 10μM increased frequency by 1744 ± 614% (n=4). Experimental traces are shown in figure 3.3. In the presence of granisetron (GRN; 1μM, n=5) 1μM PBG now only increased frequency by 58 ± 15%. Experimental traces are shown in figure 3.6. These values were significantly different to time-matched control (n=6) were frequency only changed by -2.8 ± 5.4% over the same interval. Combined data is shown in figure 3.7A. The peak effect of PBG after bath application is typically reached within 2-3 minutes (figure 3.5).

mEPSC amplitude was increased by 18 ± 5% and 42 ± 20% by applications of 1μM (n=7) and 10μM (n=4) PBG, respectively (figure 3.5A). These values were significantly different to time-matched control (n=6) where amplitude changed by -3.3 ± 3.3% over the same interval. In the presence of granisetron (1μM, n=5, figure 3.6 and 3.5B) 1μM PBG had no effect on amplitude (-3.5 ± 2.9%). Combined data is shown in figure 3.7B.

Effect of 5-HT_3 antagonist granisetron

Bath application of the 5-HT_3 granisetron (1 μM; n=6) caused a gradual and significant decline in frequency of spontaneous mEPSCs (figure 3.8A and 3.9A). The effect reached its maximum of -36 ± 4% between 5 to 7 min following application (figure 3.8B). Granisetron had no significant effect on mEPSC amplitude (figure 3.9B).

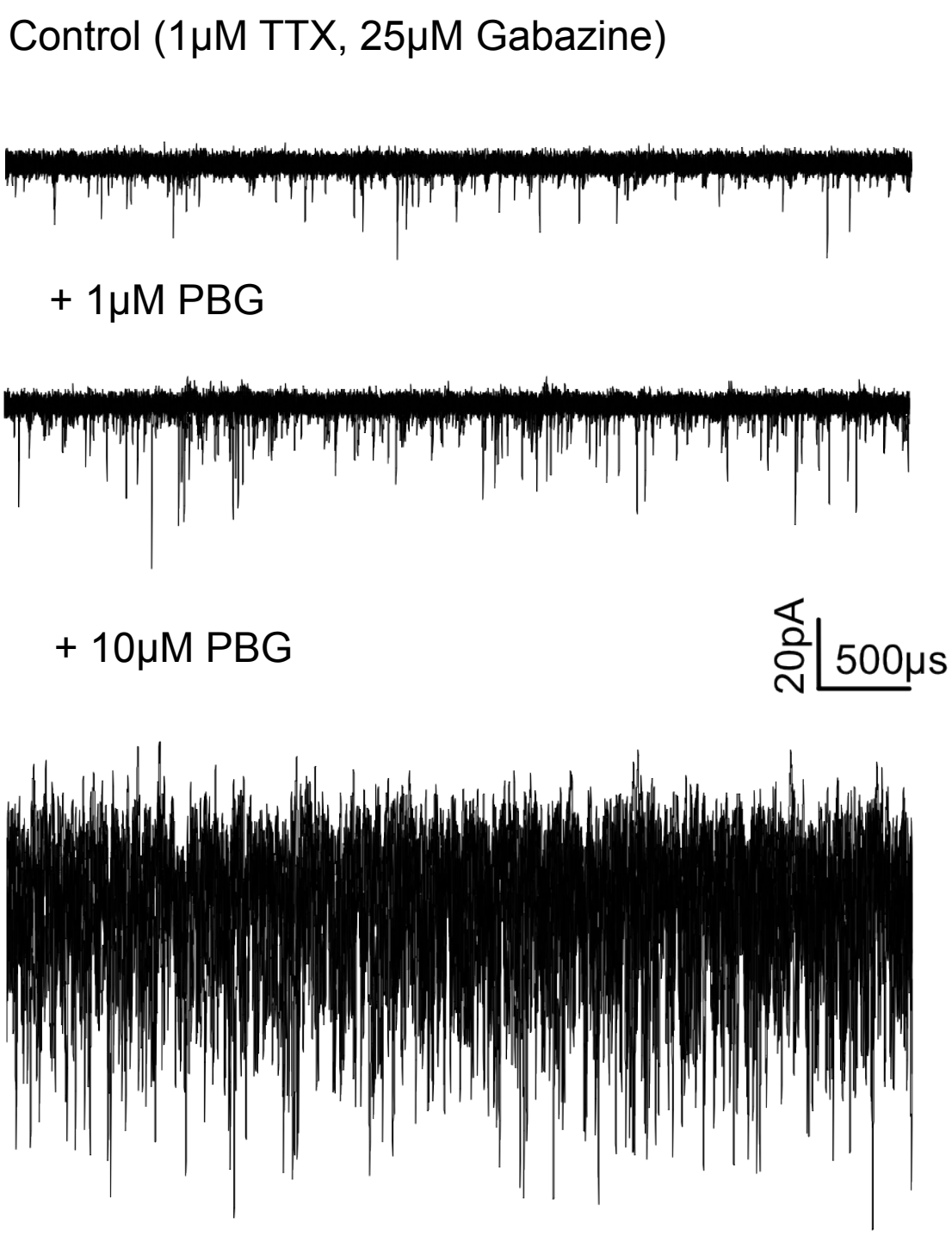


Figure 3.3 Effect of PBG on mEPSCs: Traces

Representative mEPSC traces from a neuron recorded in the NTS during control (aCSF + 1µM TTX and 25µM gabazine) and following bath applications of phenylbiguanide (PBG; 1 and 10µM) . Each panel represents 6 overlapping traces 5s in length.

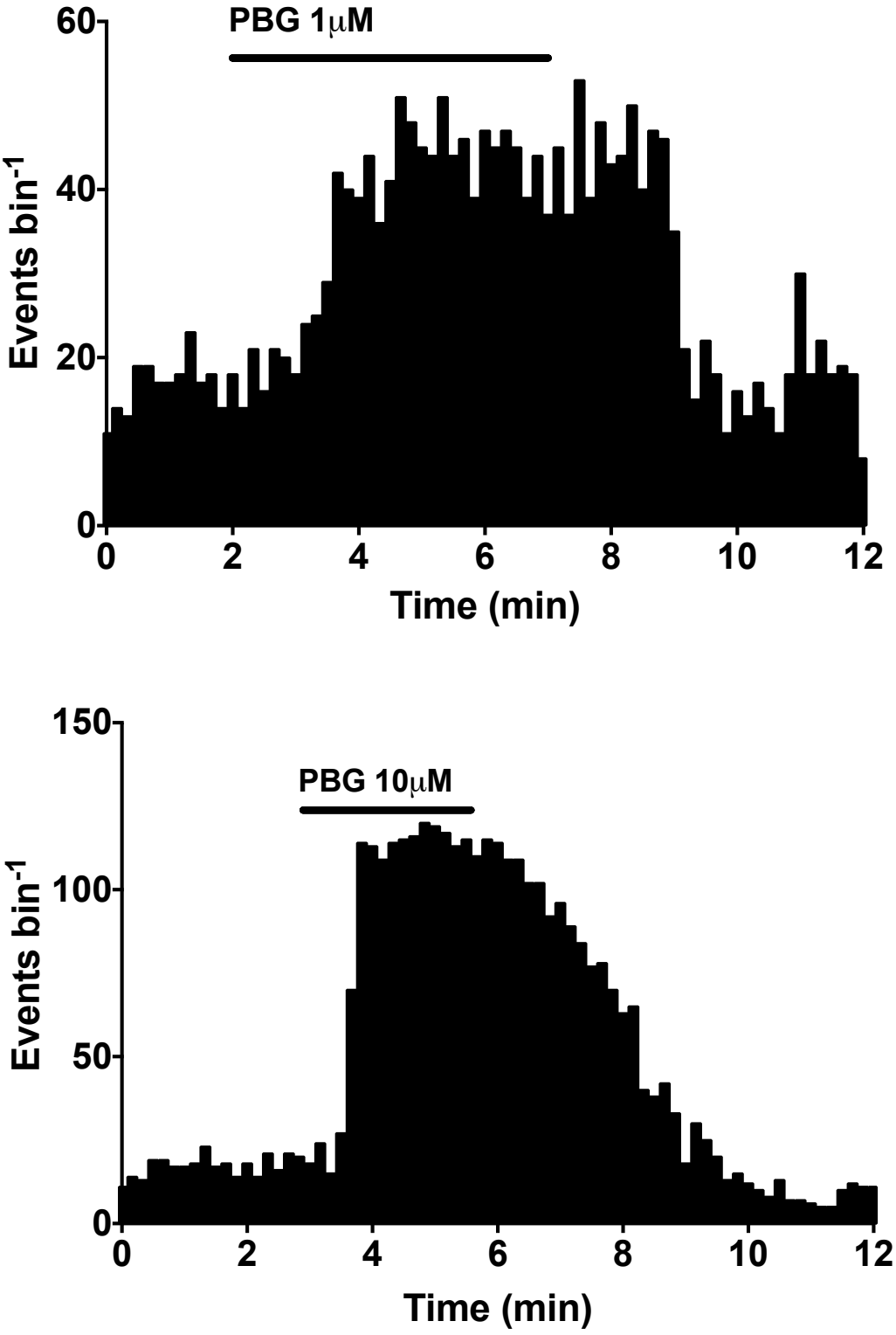


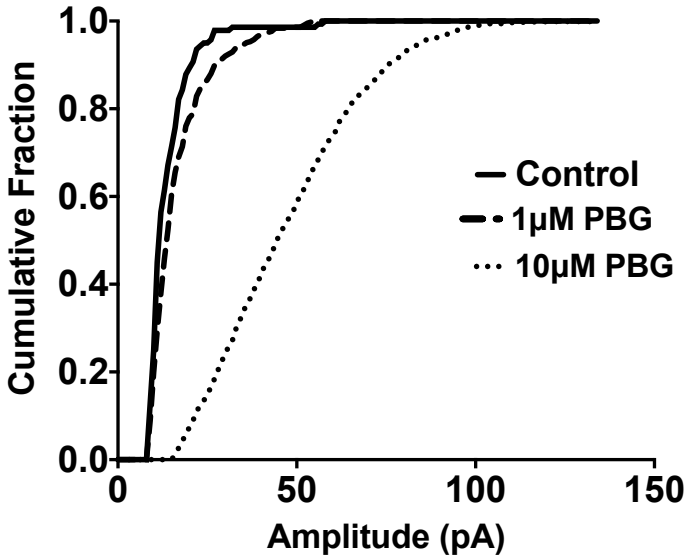
Figure 3.4 Effect of PBG on mEPSCs: Frequency Histogram

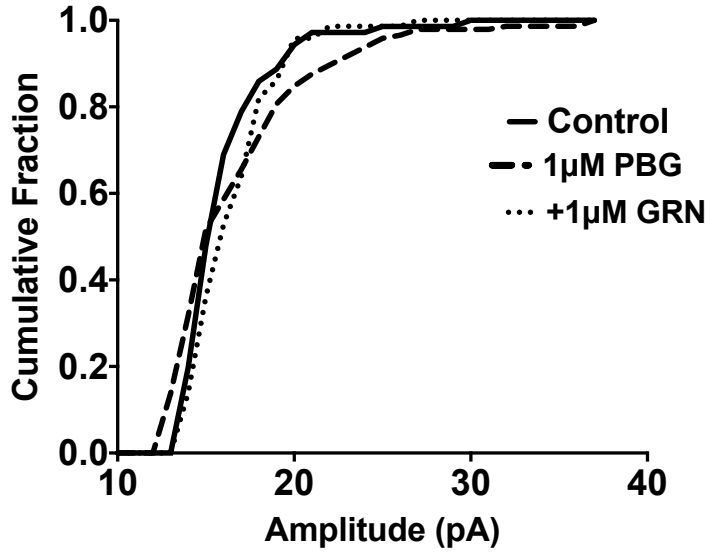
Frequency histograms of average mEPSC frequency over time binned into 10s periods from a neuron recorded in the NTS treated with 1μM (*upper)* and 10μM (*lower-note difference in scale*) phenylbiguanide (PBG).

Figure 3.5 Effect of PBG on mEPSCs: Amplitude

A) Cumulative fraction of mEPSC event amplitudes during control and following bath applications of phenylbiguanide (PBG; 1-10μM). Events were cumulatively binned at 1pA intervals.

B) Cumulative fraction of mEPSC event amplitudes during control and following bath applications of phenylbiguanide (PBG; 1μM) and PBG + granisetron (GRN). Events were cumulatively binned at 1pA intervals.


Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
Amplitude (pA)
Control
1μM PBG
10μM PBG


Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
Amplitude (pA)
Control
1μM PBG
+1μM GRN

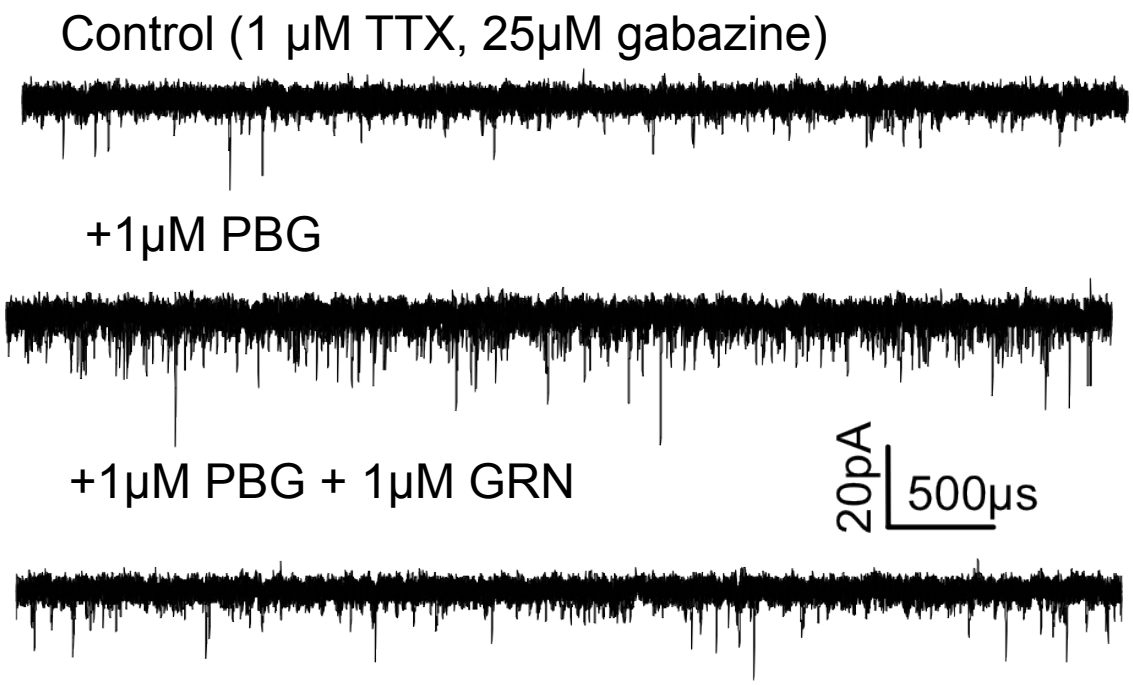


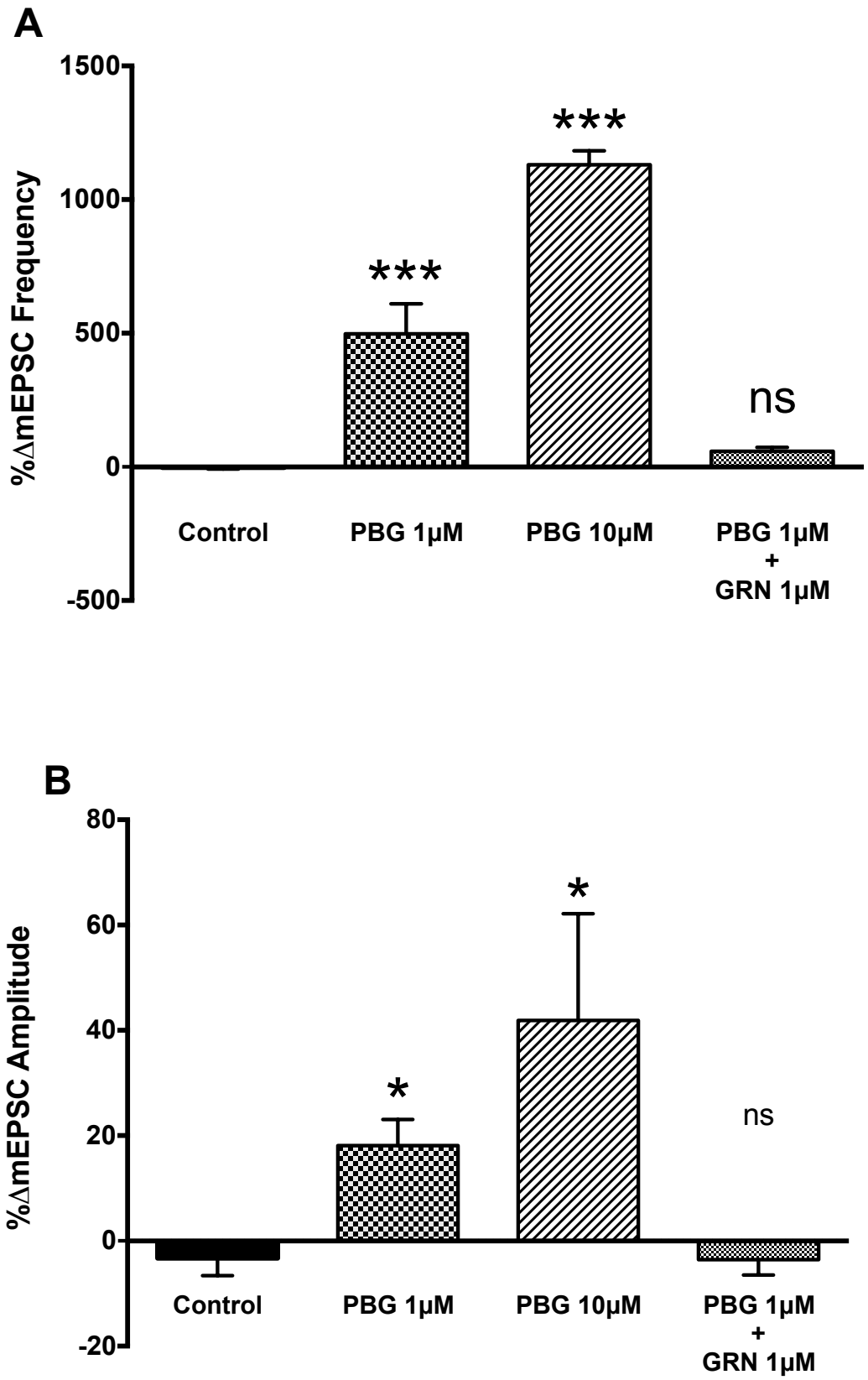
Figure 3.6 Attenuation of the effect of PBG with granisetron

A) Representative mEPSC traces from a neuron recorded in the NTS during control (aCSF + 1μM TTX and 25μM gabazine) and following bath applications of phenylbiguanide (PBG; 1μM) and 1μM PBG + 1μM granisetron (GRN). Each panel represents 6 overlapping traces 5s in length.

Figure 3.7 PBG and granisetron: Combined data

A) Histogram of mean (±s.e.m) changes in mEPSC frequency of NTS cells following bath application of phenylbiguanide (PBG; 1 and 10μM; n=7 and 3, respectively) in the presence and absence of granisetron (GRN; 1μM, n=5) compared to control (n=7). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; ***P<0.001, ns; non-significant.

B) Histogram of mean (±s.e.m) changes in mEPSC amplitude of NTS cells following bath application of phenylbiguanide (PBG; 1 and 10μM; n=7 and 3, respectively) in the presence and absence of granisetron (GRN; 1μM, n=5) compared to control (n=7). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; *P<0.05, ns; non-significant.


A
1500
1000
500
0
-500
%ΔmEPSC Frequency
***
***
ns
Control
PBG 1μM
PBG 10μM
PBG 1μM
+
GRN 1μM
B
80
60
40
20
0
-20
%ΔmEPSC Amplitude
*
*
ns
Control
PBG 1μM
PBG 10μM
PBG 1μM
+
GRN 1μM

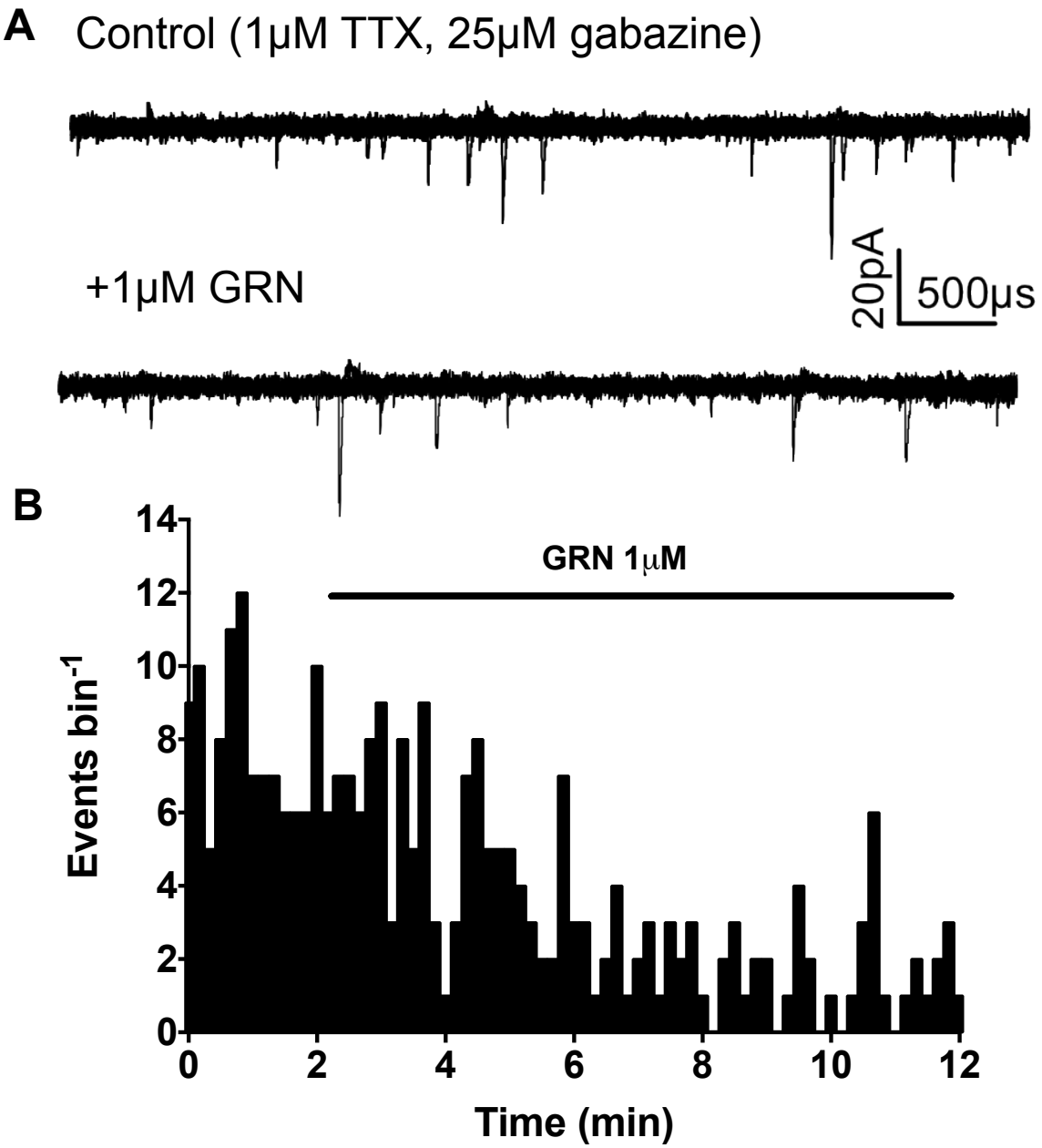


Figure 3.8 Effect of granisetron alone on mEPSC frequency

A) Representative mEPSC traces from a neuron recorded in the NTS during control (aCSF + 1μM TTX and 25μM gabazine) and following bath applications of granisetron (GRN; 1μM). Each panel represents 6 overlapping traces 5s in length. *B*) Frequency histogram of mEPSC frequency over time binned into 10s periods from a neuron recorded in the NTS treated with 1μM granisetron (GRN).

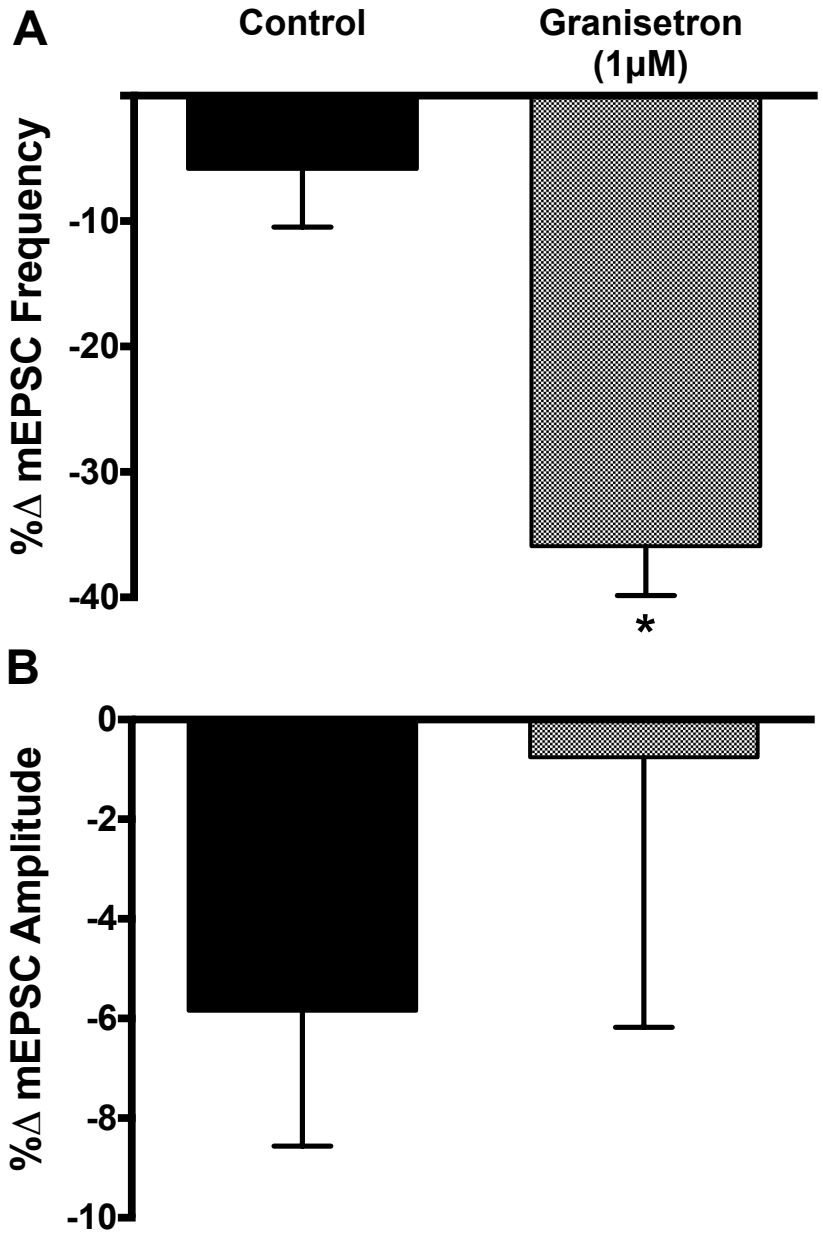


Figure 3.9 Effect of granisetron on mEPSC frequency: Combined data

Histograms of mean (±s.e.m) changes in mEPSC *A*) frequency and *B*) amplitude of NTS cells following bath application of granisetron (GRN; 1µM n=6) and aCSF (control; n=6). Compared to control with Students unpaired *t*-test; **P*<0.05.

3.5. Effect of 5-HT on spontaneous glutamate release

Effect of 5-HT on mEPSC frequency

5-HT at a range of concentrations (0.3μM - 10μM) was applied to the brainstem slice while recording mEPSC activity from a cell within the NTS. Experimental traces are shown in figure 3.10. Application of 0.3μM 5-HT decreased the frequency of spontaneous mEPSCs in all NTS neurons tested (n=5) by 53 ± 12%, while 1μM 5-HT had a mixed effect. In 6 of 11 neurons tested there was a significant decrease in frequency of 62 ± 10%. In the remaining 5 there was a significant increase in frequency of 403 ± 142%. Application of 10μM 5-HT significantly increased mEPSC frequency in all cases (n=5) by 1285 ± 276%. Application of 100μM 5-HT significantly increased mEPSC frequency in all cases (n=5) by 2462 ± 543%. Combined data of inhibitory effects is shown in figure 3.13 and excitatory effects are shown in figure 3.14 for clarity.

Time of onset for both excitatory and inhibitory peak responses was approximately 4-5 minutes. In both cases there was a gradual increase in drug effect to plateau starting approximately 1minute after the drug was introduced to the slice bath (figure 3.11).

Concentrations of 0.3 and 1μM 5-HT in cells that had an inhibitory effect mEPSC frequency also had no effect on mEPSC amplitude. In cells that displayed an excitatory effect on mEPSC frequency, amplitude was significantly increased (c.f. control; -3.2 ± 3.3%) by 21.4 ± 4.8% and 51.7 ± 8.5% when the slice was exposed to 5-HT concentrations of 10μM and 100μM. However, 1μM 5-HT had no significant effect on mEPSC amplitude (-2.7 ± 5.0%; figure 3.12). Combined data is shown in figure 3.15.

Effect of 5-HT_{1A} and 5-HT_3 antagonists

In the presence of granisetron (1μM, n=6), the highest dose of 5-HT now caused a decrease in mEPSC frequency of 30±11 %. This was in addition to the inhibitory effect of granisetron alone (see section 3.4). The lowest dose (0.3μM), in the presence of the 5-HT_{1A} antagonist WAY-10035 now caused no change in mEPSC frequency of 3.3 ± 22.3% (n=8). In both cases there was no significant effect on mEPSC amplitude. Combined data is shown in figure 3.14.

Figure 3.10 Effect of 5-HT on mEPSC frequency: Traces

Representative mEPSC traces from neurons recorded in the NTS during control (aCSF + 1μM TTX and 25μM gabazine) and following bath applications of 5-HT (0.3-100μM). Each panel represents 6 overlapping traces 5s in length.

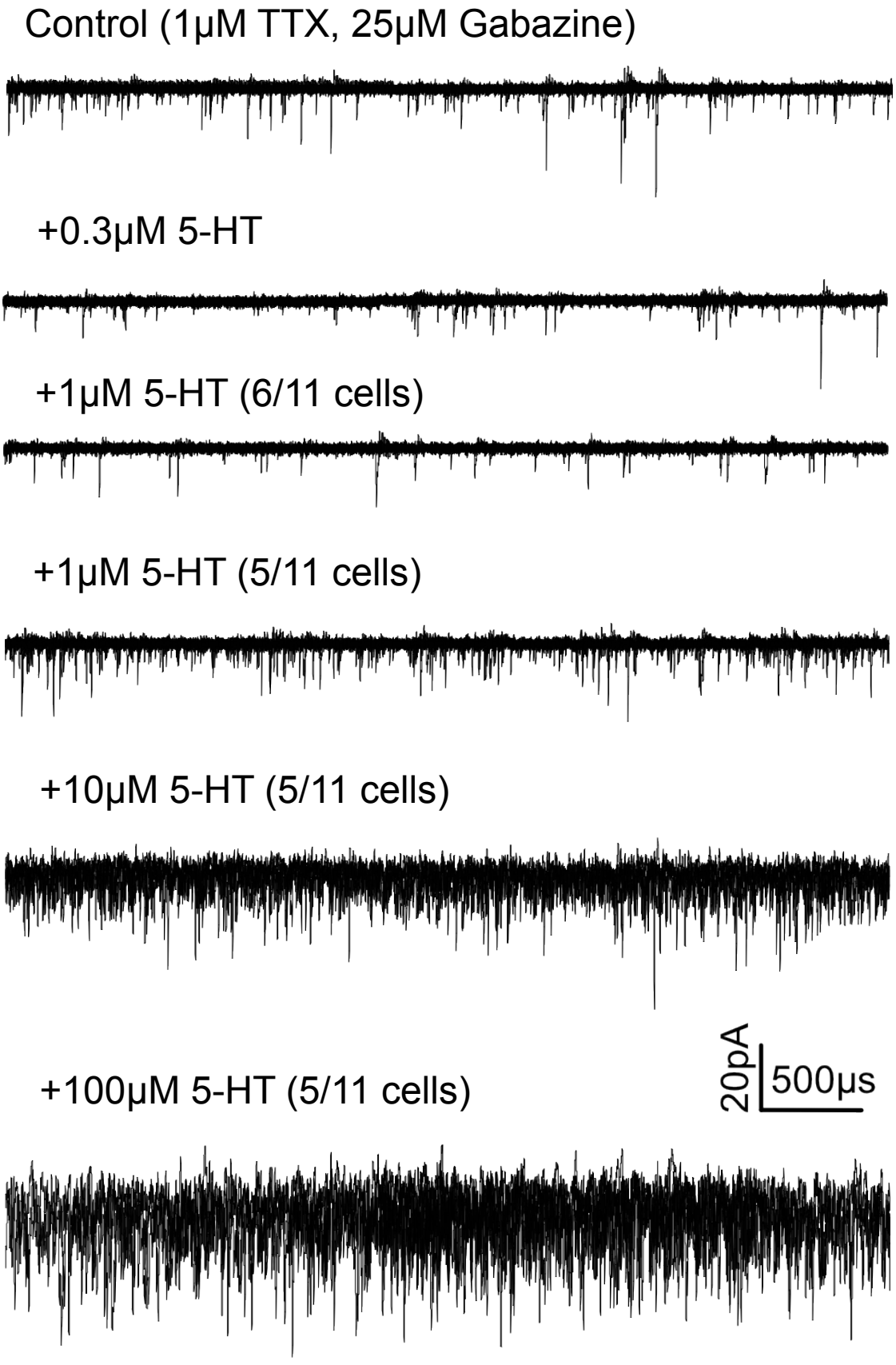
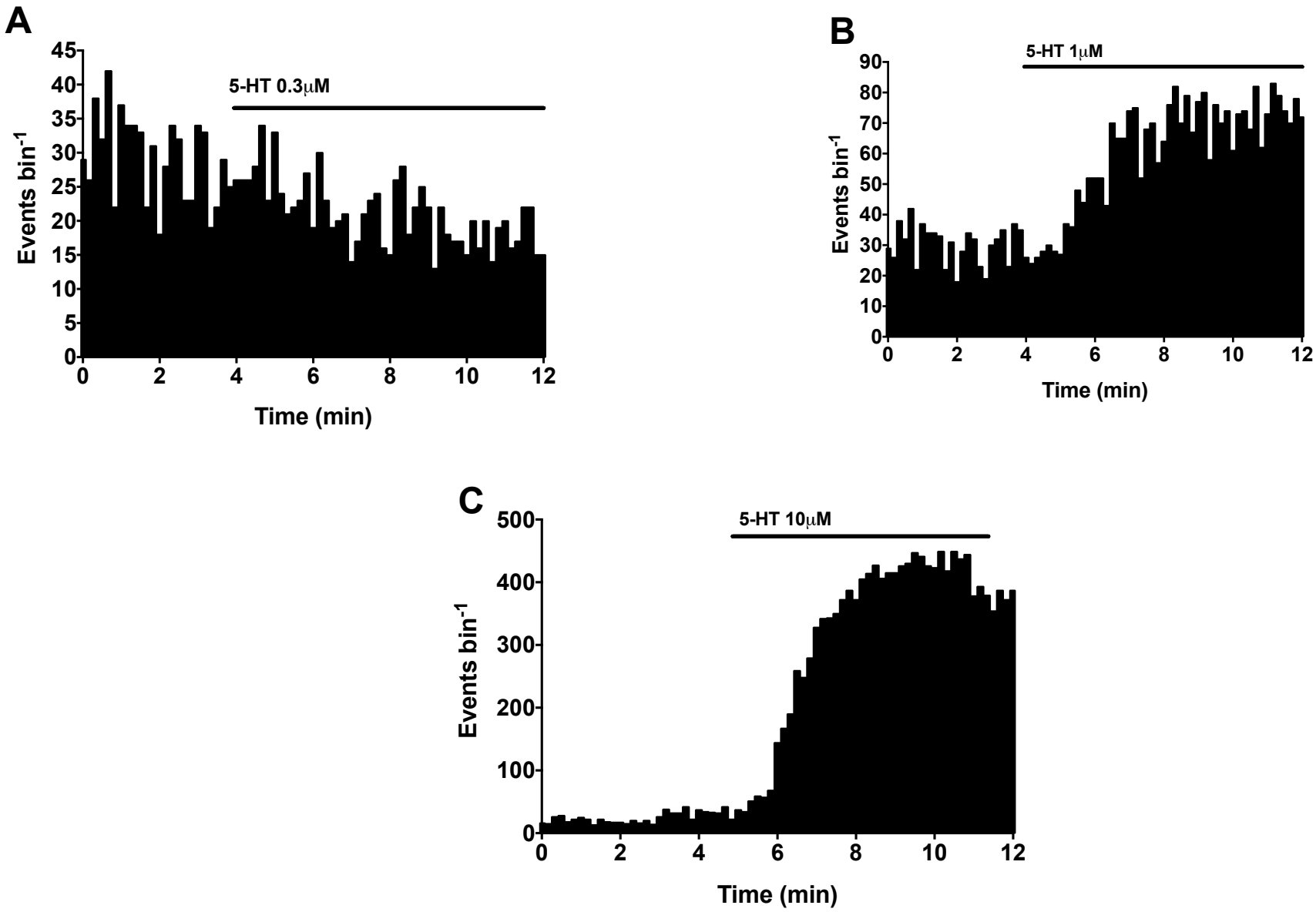

Control (1μM TTX, 25μM Gabazine)
+0.3μM 5-HT
+1μM 5-HT (6/11 cells)
+1μM 5-HT (5/11 cells)
+10μM 5-HT (5/11 cells)
+100μM 5-HT (5/11 cells)
20pA
500μs

Figure 3.11 Effect of 5-HT on mEPSC frequency: Frequency Histograms

Frequency histogram of mEPSC frequency over time binned into 10s periods from 3 neurons recorded in the NTS treated with A) 0.3μM B) 1μM and C) 10μM 5-HT.


A
5-HT 0.3μM
Events bin-1
Time (min)
B
5-HT 1μM
Events bin-1
Time (min)
C
5-HT 10μM
Events bin-1
Time (min)

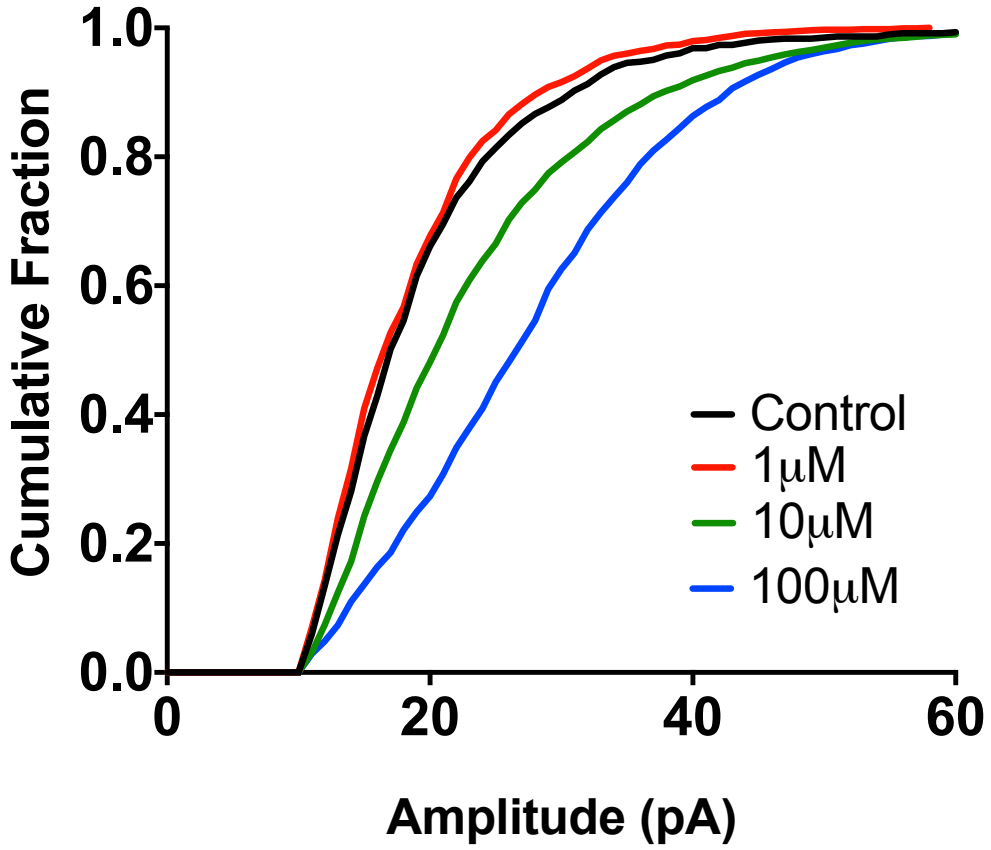


Figure 3.12 Effect of 5-HT on amplitude: Cumulative fraction plot

Cumulative fraction plot of mEPSC amplitudes from the same cell during control (aCSF + 1μM TTX and 25μM gabazine) and following bath applications of 5-HT 1-100 μM. Events were cumulatively binned at 1pA intervals

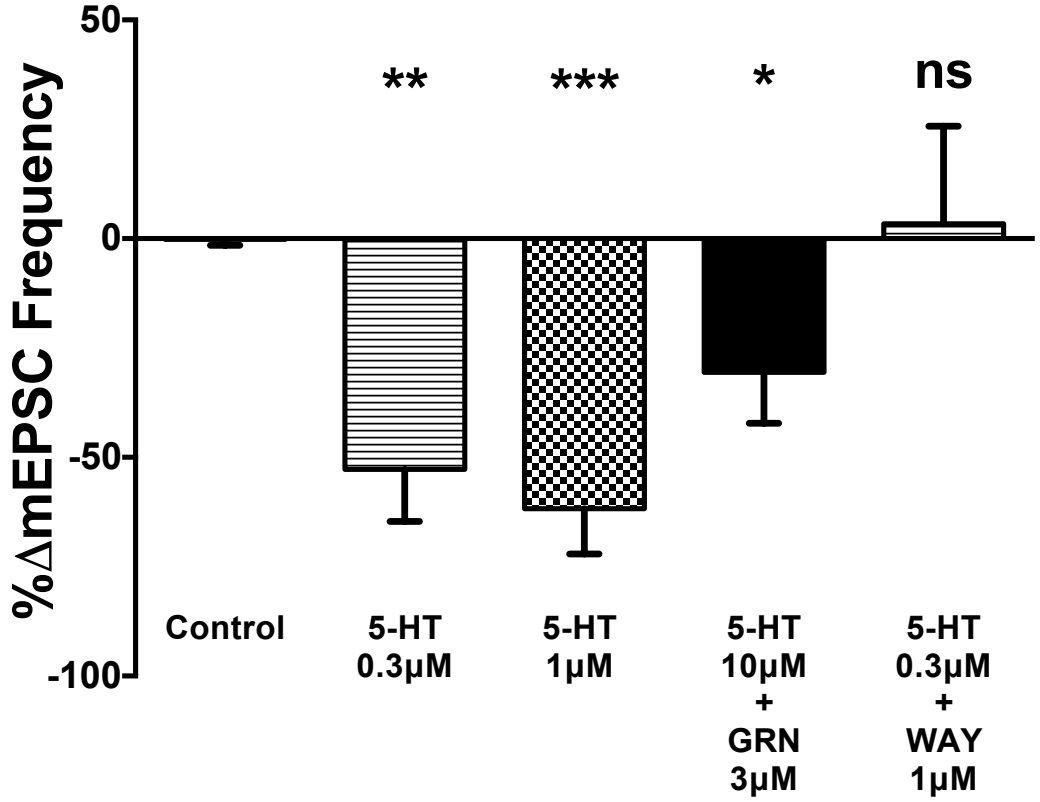


Figure 3.13 Effect of low dose 5-HT on frequency: Combined Data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC frequency of NTS cells following bath application of aCSF (Control: aCSF + 1µM TTX and 25µM gabazine; n=6), 0.3µM (n=5), 1µM 5-HT (n=5). Additionally, 10µM 5-HT in the presence of granisetron (GRN; 3µM n=6) and 0.3µM 5-HT in the presence of WAY-100635 (WAY; 1µM (n=8). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; *P<0.05, **P<0.01, *** P<0.001, ns; non-significant.

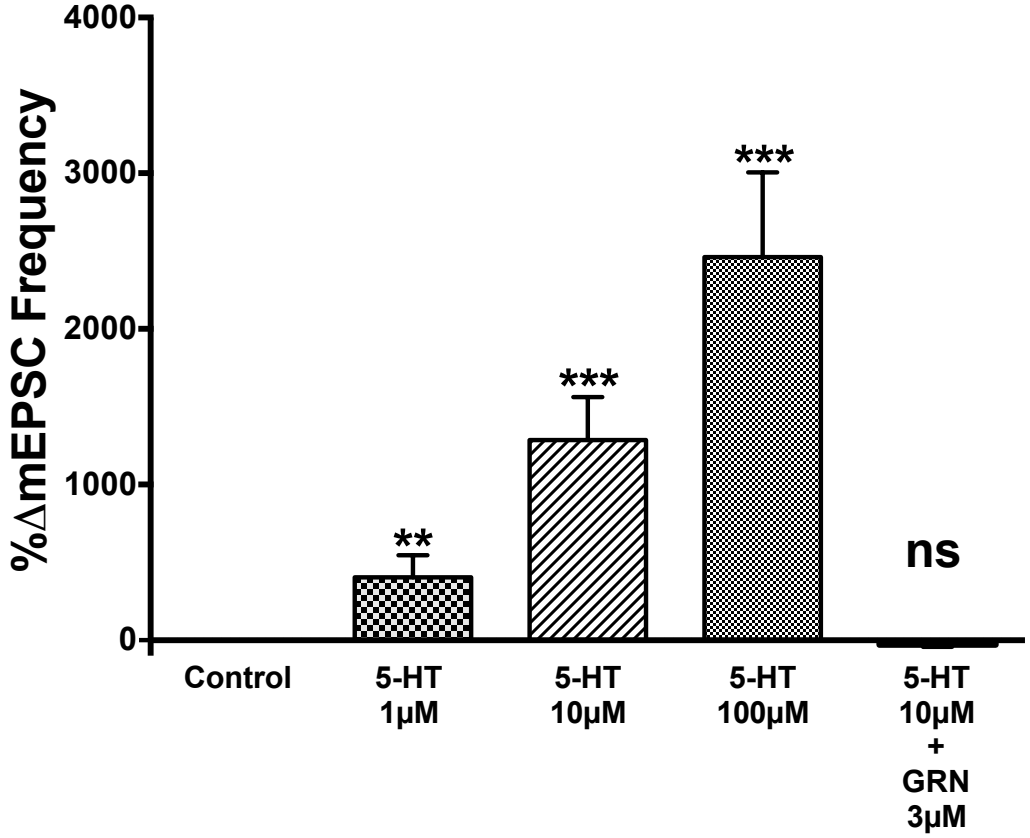


Figure 3.14 Effect of high dose 5-HT on frequency: Combined Data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC frequency of NTS cells following bath application of aCSF (Control: aCSF + 1μM TTX and 25μM gabazine; n=6), 0.3μM (n=5), 1μM 5-HT (n=5). Additionally, 10μM 5-HT in the presence of granisetron (GRN; 3μM n=6). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; ** $P<0.01$, *** $P<0.001$, ns; non-significant.

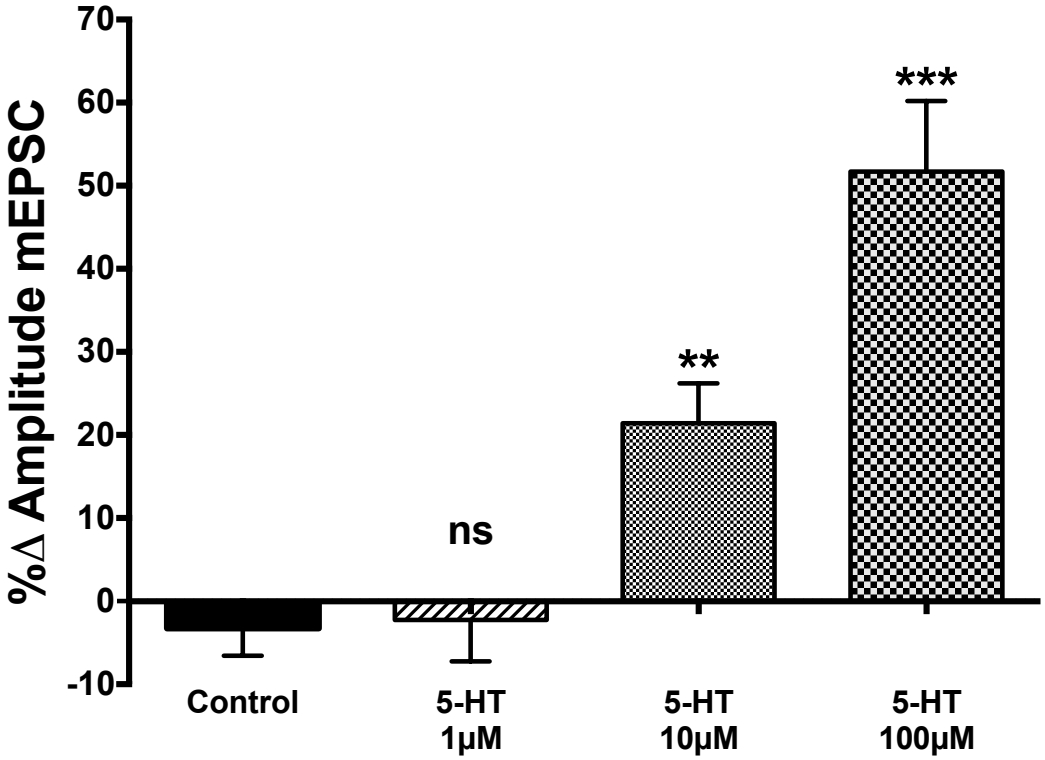


Figure 3.15 Effect of 5-HT on mEPSC amplitude: Combined data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC amplitude of NTS cells following bath application of aCSF (Control: aCSF + 1μM TTX and 25μM gabazine; n=6), 1μM 5-HT (n=5), 10μM 5-HT (n=6) and 100μM (n= 5). Data for 1μM 5-HT taken from those cells excited by 5-HT application. Compared to control with 1-Way ANOVA followed by Fisher's LSD test; ** $P<0.01$, *** $P<0.001$, ns; non-significant.

3.6. Effect of re-uptake inhibitors on spontaneous glutamate release

Experimental traces showing the effect of the various re-uptake inhibitors are shown in figure 3.16. Combined data of the effects of citalopram, the 5-HT transporter inhibitor, alone and in the presence WAY-100635 is shown in figure 3.17. The effects of decynium-22, the 5-HT OCT3/PMAT inhibitor, alone and in the presence WAY-100635 is shown in figure 3.18

Effect of 5-HT transporter inhibitor citalopram

Application of the 5-HT transporter inhibitor citalopram (500nM; n=5) caused a significant decrease of 54 ± 5% in the spontaneous frequency of mEPSCs, whilst a higher dose of citalopram (5 μM; n=5) had a similar effect (-38 ± 6%). These values were significantly different to the time-matched control (n=7) where frequency changed by -2.5 ± 4.4% over the same interval. In both cases there was no significant effect on mEPSC amplitude.

Effect of OCT_3/PMAT inhibitor D-22

Similarly blockade of OCT3/PMAT with D-22 1μM (n=4) also caused a significant reduction in mEPSCs of 33 ± 4%, while 10μM (n=3) caused a reduction of 44 ± 6%. In all cases amplitudes remained unchanged. Again, these values were significantly different to the time-matched control over the same interval. In both cases there was no significant effect on mEPSC amplitude.

Effect of 5-HT_{1A} antagonists on uptake blockade

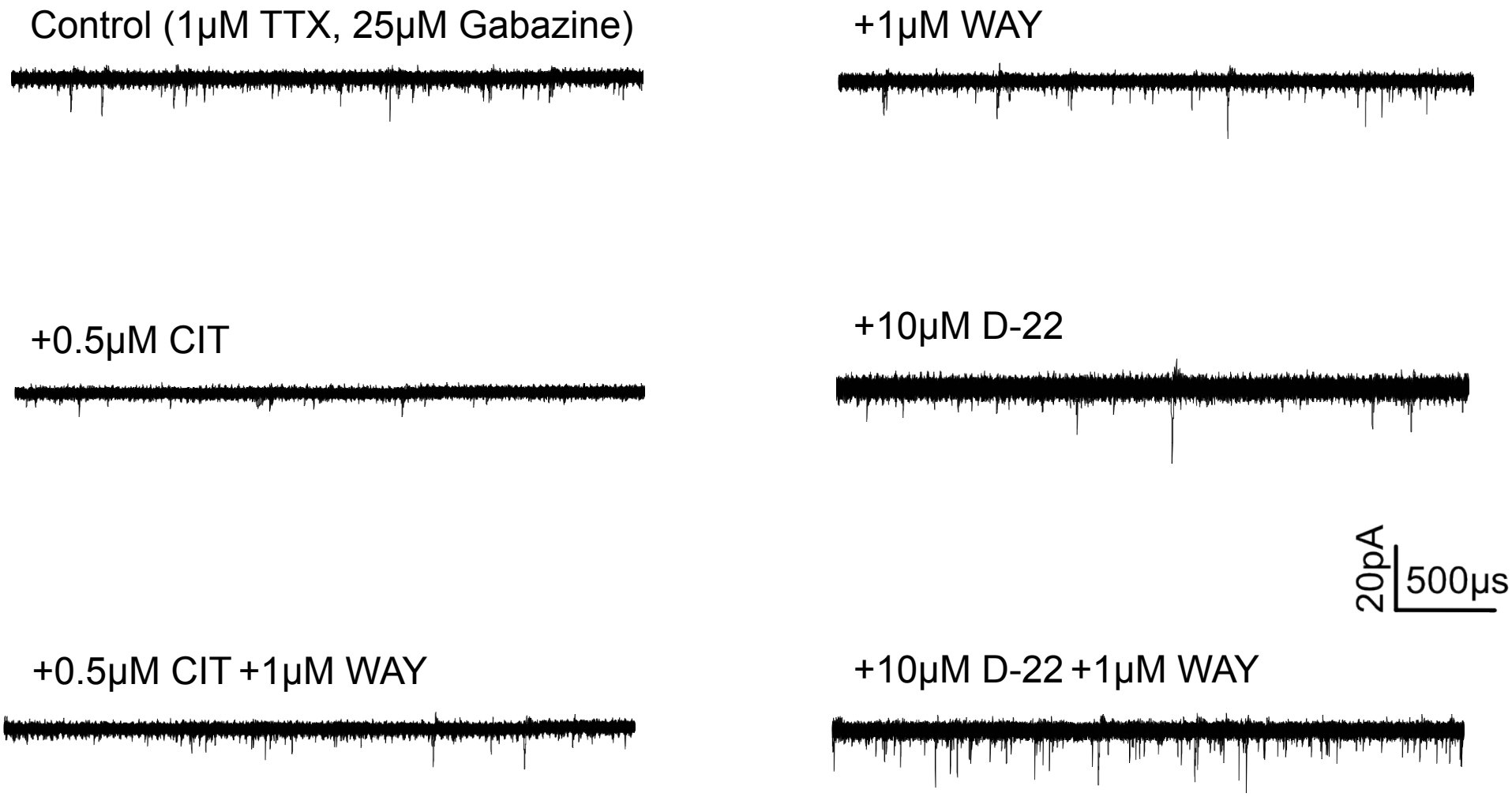
Application of aCSF (control; n=11) and WAY-100635 (1μM; n=12) and no significant effect on the spontaneous mEPSC frequency changing by -7.2 ± 2% and 1.2 ± 4%, respectively. However pre-treatment with WAY-100635 blocked the ability of both doses of citalopram (0.5 μM; n=5, 5 μM; n=4) to significantly reduce mEPSC frequency now only changing -12 ± 5% and 1.8 ± 23%, respectively. WAY-100635 pre-treatment also blocked the ability of both doses of D-22 (1μM; n=6; 10μM; n=4) to decrease mEPSC frequency, although for the higher dose of D-22 a significant increase in mEPSC frequency (57 ± 39%) was now observed. In all cases amplitudes remained unchanged.

Effect of corticosterone

Application of the OCT3 inhibitor corticosterone (1μM; n=5; 3μM; n=4) caused no change in mEPSC frequency at both doses. After application of the lower dose mEPSC frequency changed by -1.5 ± 22% and the higher dose caused change of 10 ± 19%. Both were found to be not significantly different from control (figure 3.19). Again, amplitude remained unchanged.

Figure 3.16 Effect of re-inhibitors on mEPSC frequency: Traces

Representative mEPSC traces from neurons recorded in the NTS during control (aCSF + 1μM TTX and 25μM gabazine) and following bath applications of citalopram (CIT; 0.5μM), Decynium-22 (D-22; 10μM), WAY-100635 (WAY; 1μM) and citalopram and D-22 in the presence of WAY-100635. Each panel represents 6 overlapping traces 5s in length.


Control (1μM TTX, 25μM Gabazine)
+1μM WAY
+0.5μM CIT
+10μM D-22
20pA
500μs
+0.5μM CIT +1μM WAY
+10μM D-22 +1μM WAY

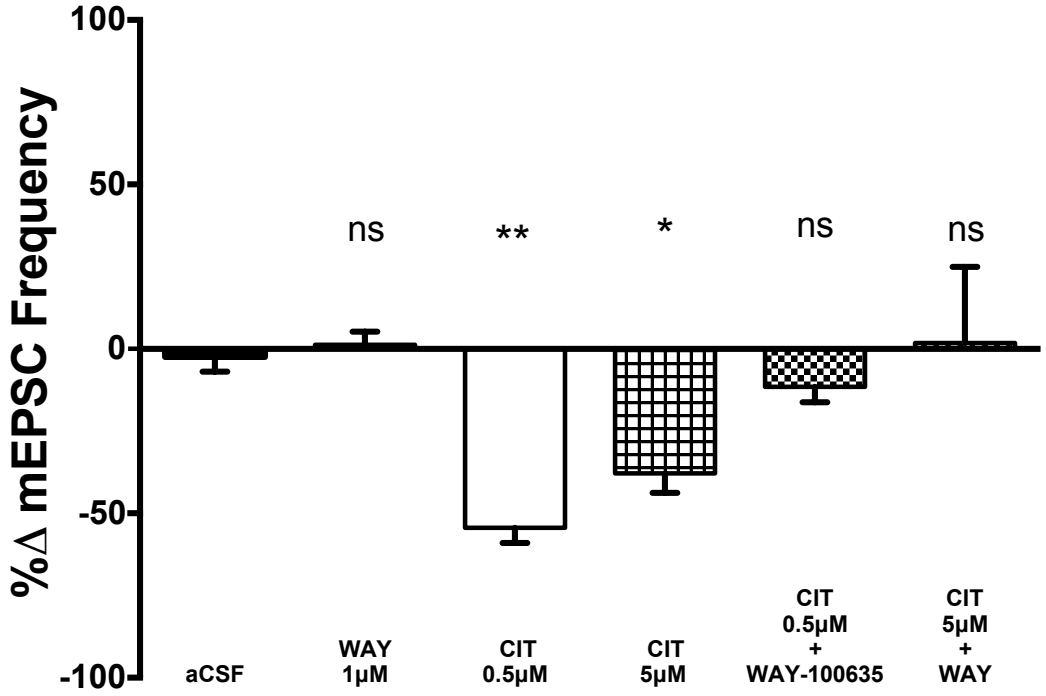


Figure 3.17 Re-uptake inhibitors: Citalopram combined data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC frequency of NTS cells following bath application of aCSF (Control: aCSF + 1µM TTX and 25µM gabazine; n=7), WAY-100635 (WAY; 1µM, n=12), citalopram (CIT; 0.5µM, n=5 & 5µM, n= 5) and citalopram in the presence of 1µM WAY-100635 (0.5µM; n=6, 5µM; n=4). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; *P<0.05, **P<0.01, ns; non-significant.

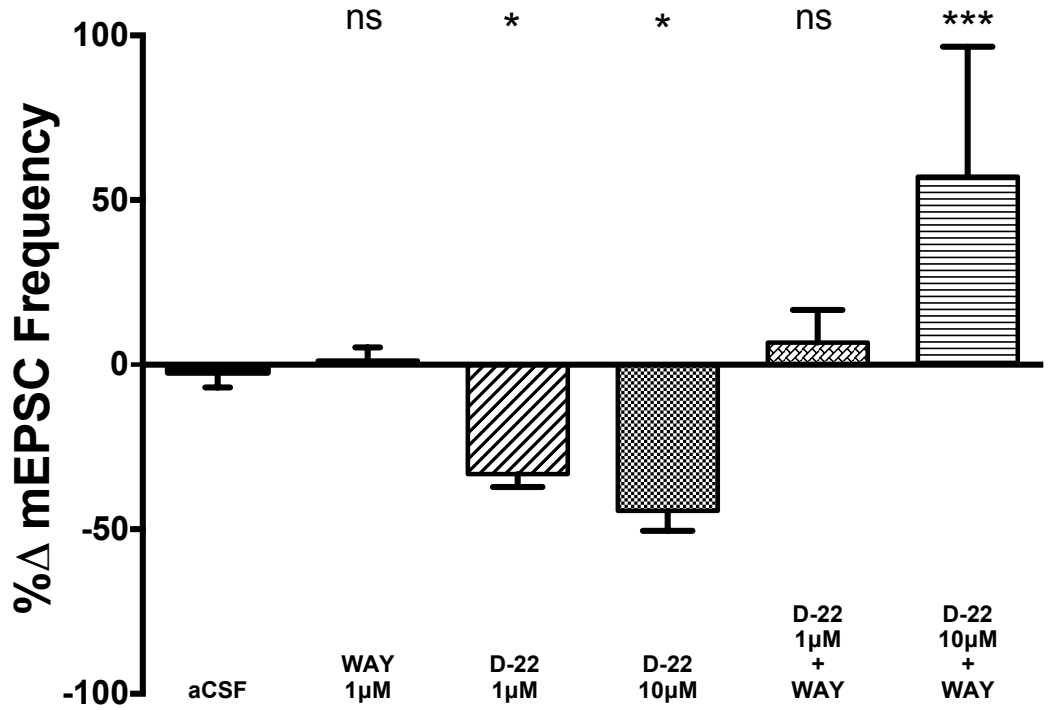


Figure 3.18 Re-uptake inhibitors: D-22 combined data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC frequency of NTS cells following bath application of aCSF (Control: aCSF + 1μM TTX and 25μM gabazine; n=7), WAY-100635 (WAY; 1μM, n=12), Decynium-22 (D-22; 1μM, n=4 & 10μM, n= 3) and Decynium-22 in the presence of 1μM WAY-100635 (1μM; n=6, 10μM; n=4). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; *P<0.05, *** P<0.001, ns; non-significant.

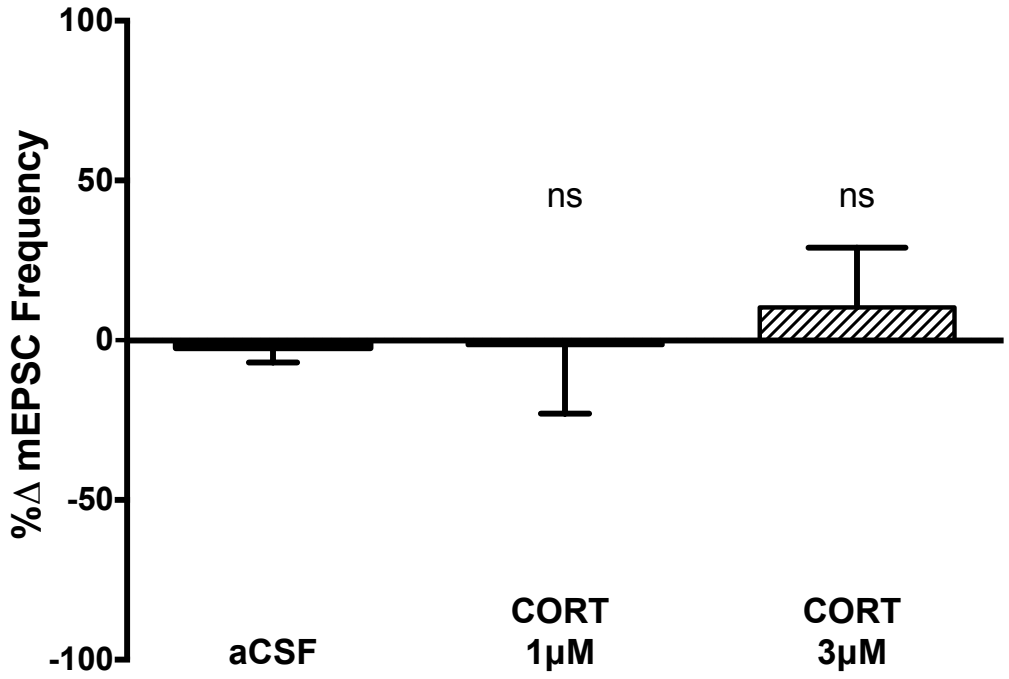


Figure 3.19 Effect of corticosterone: combined data

Histogram of mean (±s.e.m) percentage changes over baseline in mEPSC frequency of NTS cells following bath application of aCSF (Control: aCSF + 1µM TTX and 25µM gabazine; n=7), corticosterone (CORT; 1µM, n=5 & 3µM, n= 4). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; ns; non-significant.

3.7. Effect of re-uptake inhibitors on stimulated glutamate release

The effect of transporter inhibitors on synaptic transmission was assessed in 26 further NTS neurons in which EPSCs were evoked by electrical stimulation of the solitary tract (TS) in the present of gabazine (25 μM) alone. Application of CNQX (10mM n=4) completely abolished in evoked inward current (figure 3.20).

Effect of 5-HT_3 receptor antagonist granisetron

Granisetron (3 μM; n=6) decreased the amplitude of the first evoked EPSC when compared to control; -105±6 cf. -84±10 pA (figure 3.21). Percentage change data showed that granisetron significantly decreased the amplitude of the first evoked EPSC, when compared to control (-17.4 ± 5.3% c.f. -1.7 ± 1.90). Combined data is shown in figure 3.23. Paired-pulse ratio (PPR) increased significantly from 0.64 ± 0.12 to 0.73 ± 0.12 (figure 3.24).

Effect of 5-HT transporter inhibitor citalopram

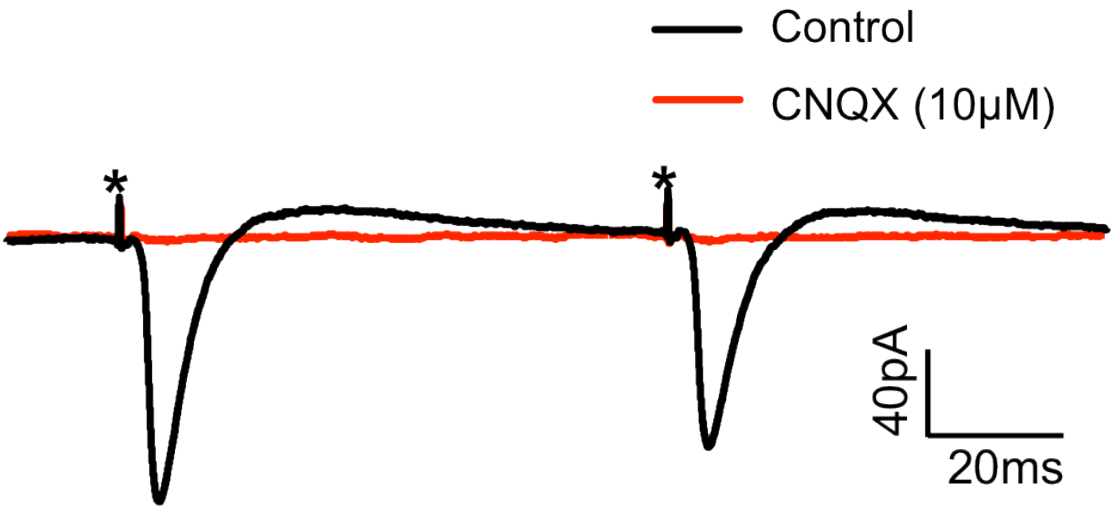
Citalopram (0.5 μM; n=10) failed to modify the amplitude of the first evoked EPSC when compared to control; -85±10 cf. -90±9 pA (figure 3.22A). Percentage change data was calculated and again, no significant change was detected between control and after drug application (-8.1 ± 6.0% c.f. -1.7 ± 1.90). Combined data is shown in figure 3.23. Paired-pulse ratio (PPR) also remained unaltered (0.66 ± 0.08 c.f. 0.68 ± 0.06). Combined data is shown in figure 3.24.

Effect of OCT_3/PMAT inhibitor D-22

D-22 (1μM; n=7) also failed to modify the evoked EPSC when compared to control; -95±12 c.f 94±7pA (figure 3.22B). Percentage change data was calculated and again, no significant change was detected between control and after drug application; -7.6 ± 5.7% c.f. -1.7 ± 1.9%. Combined data is shown in figure 3.23. Paired-pulse ratio was also unaltered (0.71 ± 0.08 c.f. 0.71 ± 0.07). Combined data is shown in figure 3.24. However, the higher dose of D-22 (10 μM; n = 4) significantly increased the amplitude of the first evoked EPSC; 107±20 c.f 131±22.6pA (figure 3.22C). Percentage change data showed significantly the amplitude was significantly increased when compared to control (27.2 ± 6.8% c.f. -1.7 ± 1.90). Paired-pulse ratio decreased significantly from 0.75 ± 0.08 to 0.44 ± 0.07 (figure 2.24).

Figure 3.20 Single pulse stimulation: effect of CNQX

Representative evoked EPSC traces from a neuron recorded in the NTS during control (aCSF + 25µM gabazine) and following bath applications of CNQX (10µM).



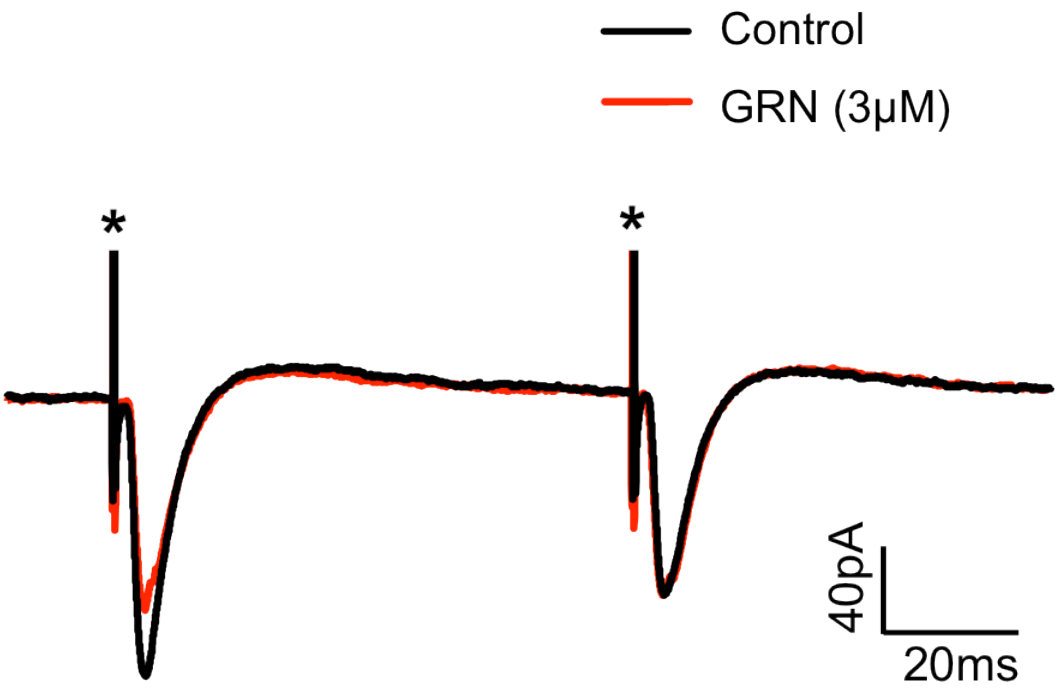


Figure 3.21 Single pulse stimulation: effect of granisetron

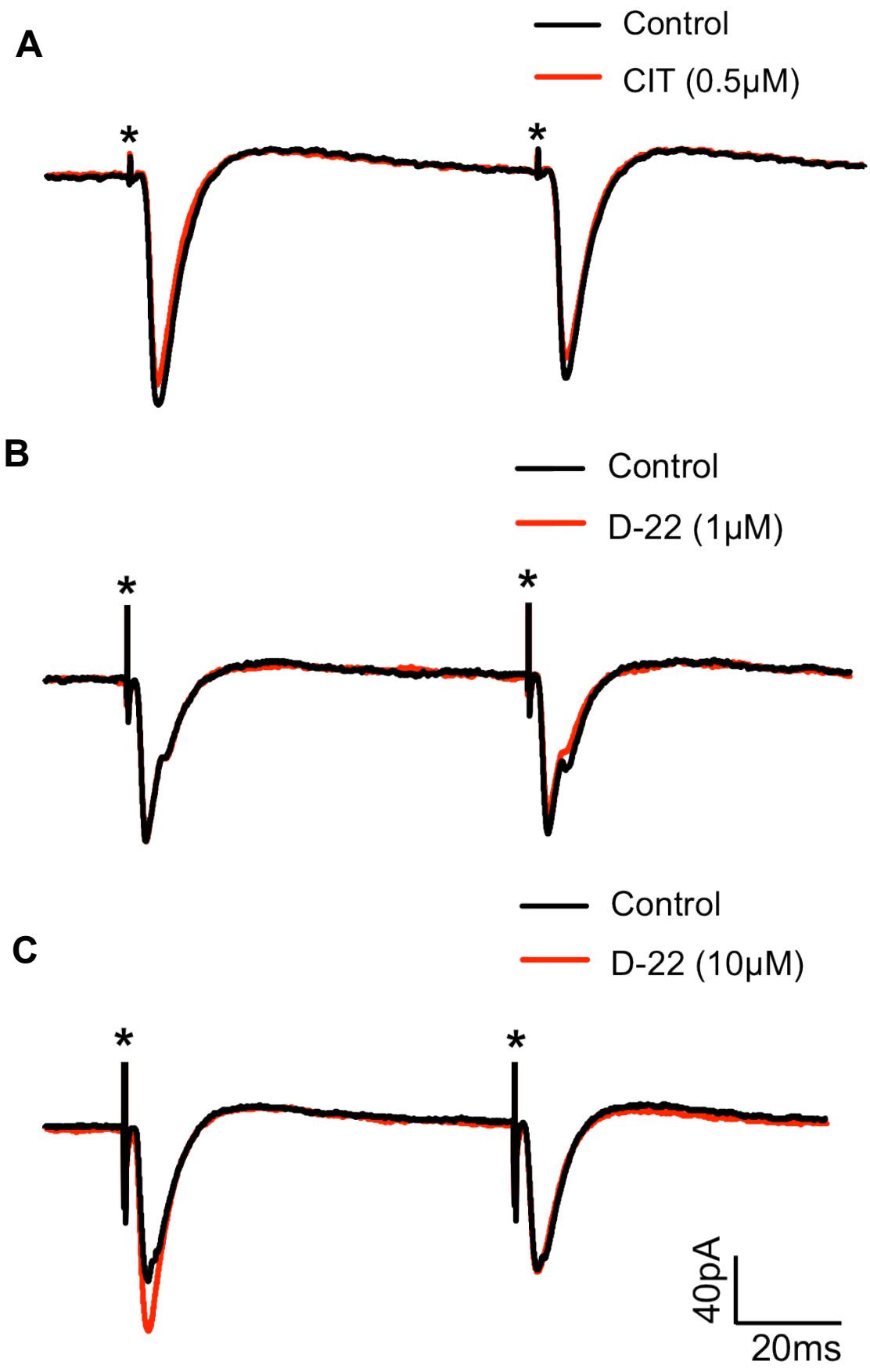
Representative evoked EPSC traces from a neuron recorded in the NTS during control (aCSF + 25μM gabazine) and following bath applications of granisetron (3μM).

Figure 3.22 Single pulse stimulation: effect of re-uptake inhibitors

A) Representative evoked EPSC traces from a neuron recorded in the NTS during control (aCSF + 25μM gabazine) and following bath applications of citalopram (CIT; 0.5μM).

B) Representative evoked EPSC traces from a neuron recorded in the NTS during control (aCSF + 25μM gabazine) and following bath applications of D-22 (1μM).

C) Representative evoked EPSC traces from a neuron recorded in the NTS during control (aCSF + 25μM gabazine) and following bath applications of D-22 (10μM).


A
Control
CIT (0.5μM)
B
Control
D-22 (1μM)
C
Control
D-22 (10μM)
40pA
20ms

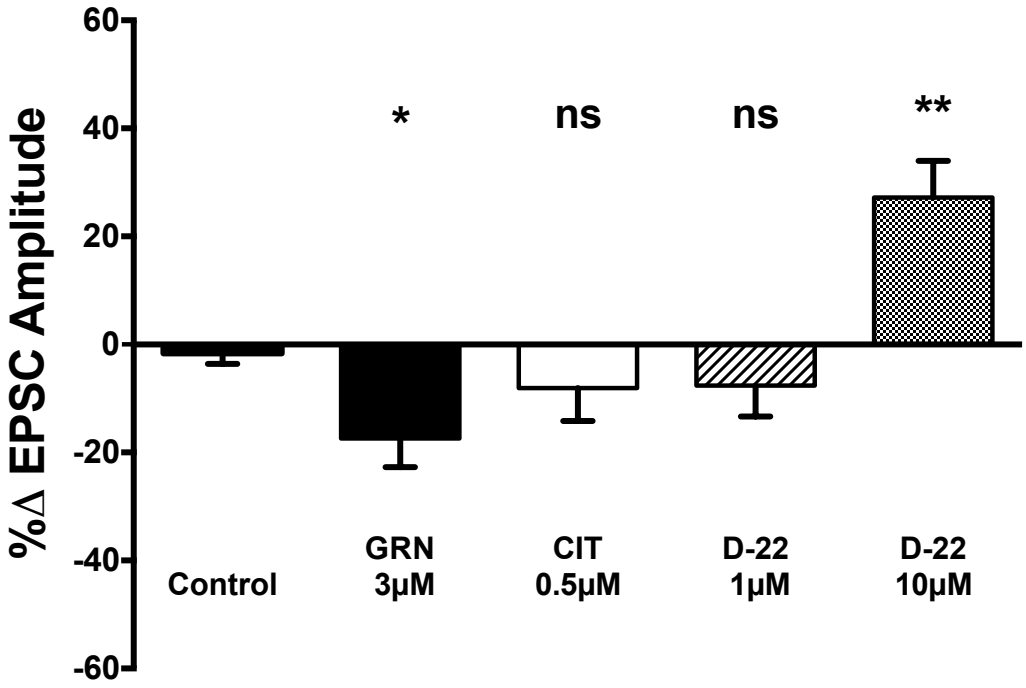


Figure 3.23 Effect of re-uptake inhibitors on EPSC amplitude: Combined data

Histogram of mean (±s.e.m) percentage changes over control in evoked EPSC amplitude recorded in NTS cells following bath application of the following: aCSF (Control: aCSF + 25μM gabazine; n=10), granisetron (GRN; 3μM, n=6), citalopram (CIT; 0.5 μM, n=10), D-22; 1μM (n=6) & 10μM (n= 4). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; *P<0.05, **P<0.01, ns; non-significant.

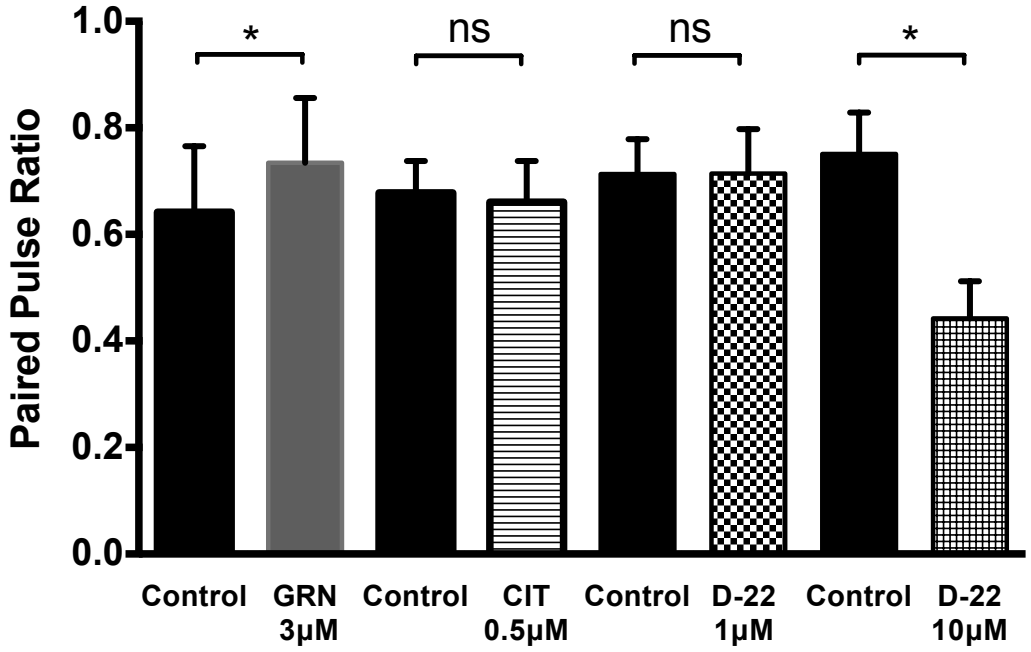


Figure 3.24 Effect on EPSC paired pulse ratio: Combined data

Histogram of mean (±s.e.m) of the amplitude ratio of a pair of EPSCs evoked by electrical tractus stimulation. Each control and drug application is taken as matched pairs from the same cell. Control: aCSF + 25µM gabazine; n=10), granisetron (GRN; 3µM, n=6), citalopram (CIT; 0.5 µM, n=10), D-22; 1µM (n=6) & 10µM (n= 4). Compared to control with Students paired t-test; *P<0.05, ns; non-significant.

Trains of 100 pulses at 20 Hz

Spontaneous EPSCs were recorded from 17 neurons. The mean frequency and amplitude were 1.6 ± 0.3 Hz and 17.1 ± 1.1pA. The frequency ranged between 0.2 to 4.6 Hz. Electrical stimulation of the TS (100 pulses at 20Hz; figure 3.25) evoked an increase in sEPSC frequency of 106 ± 13% when compared to baseline. This remained unaltered in one subsequent stimulation (100 ± 6%). Amplitude was not significantly affected. Citalopram (0.5μM; n=6) failed to alter this evoked increase in sEPSCs (99 ± 23%), however D-22 (1μM; n=8, figure 3.26) caused a significant (P<0.01) increase in sEPSCs (242 ± 20%). Amplitude was unaffected by both stimulation and drug application. Combined data is shown in figure 3.27.

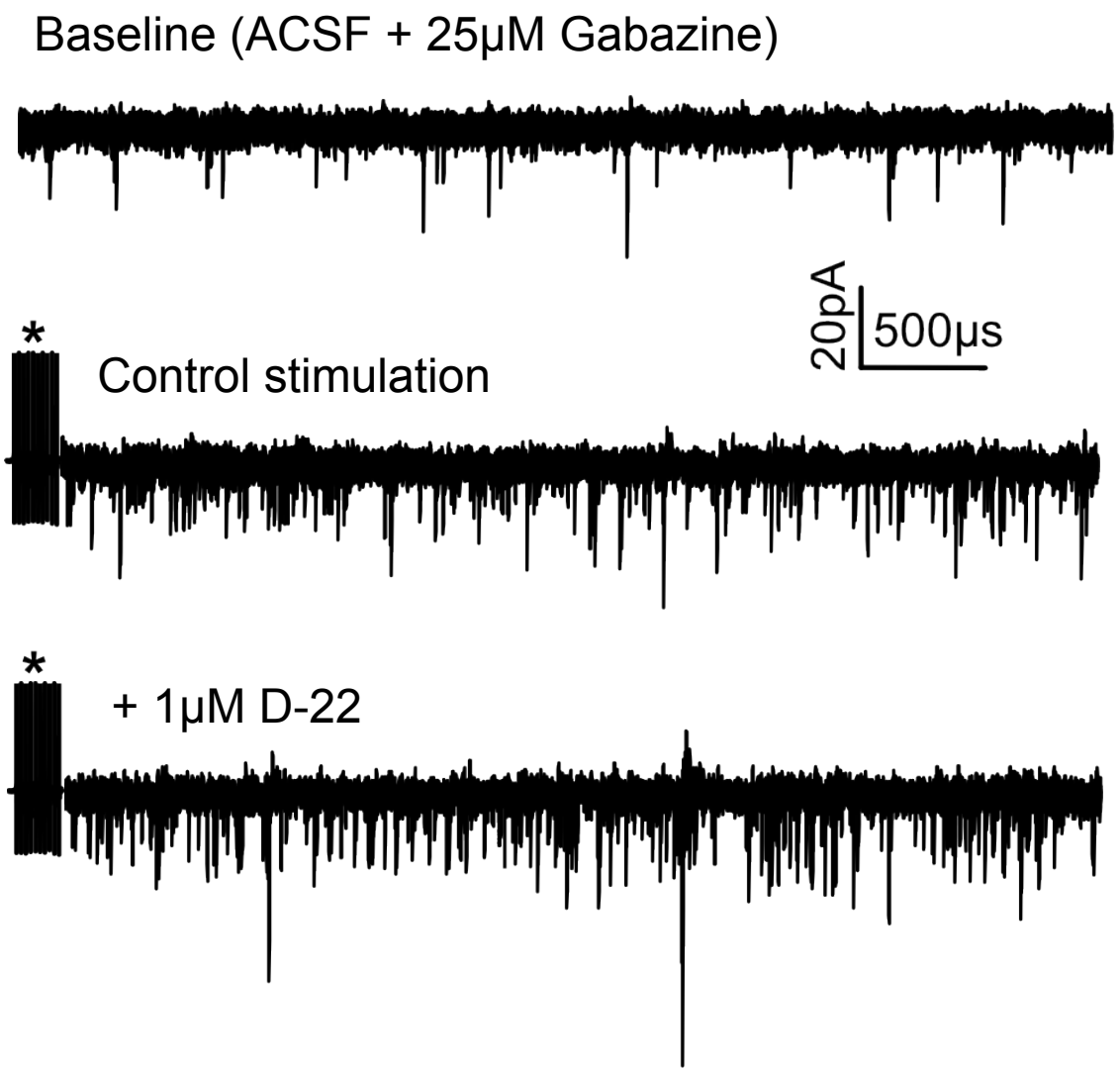


Figure 3.25 20Hz Train stimulation: Traces

Representative experimental traces of spontaneous EPCS from a neuron recorded in an NTS neuron. Baseline was recorded in ACSF with 25µM gabazine. Trains of 100 stimulation pulses were then applied to the solitary tract (*denotes stimulus artifact) in ACSF with 25µM gabazine. The stimulus was repeated 10min after application of 1µM D-22. Each panel represents 6 overlapping traces 5s in length.

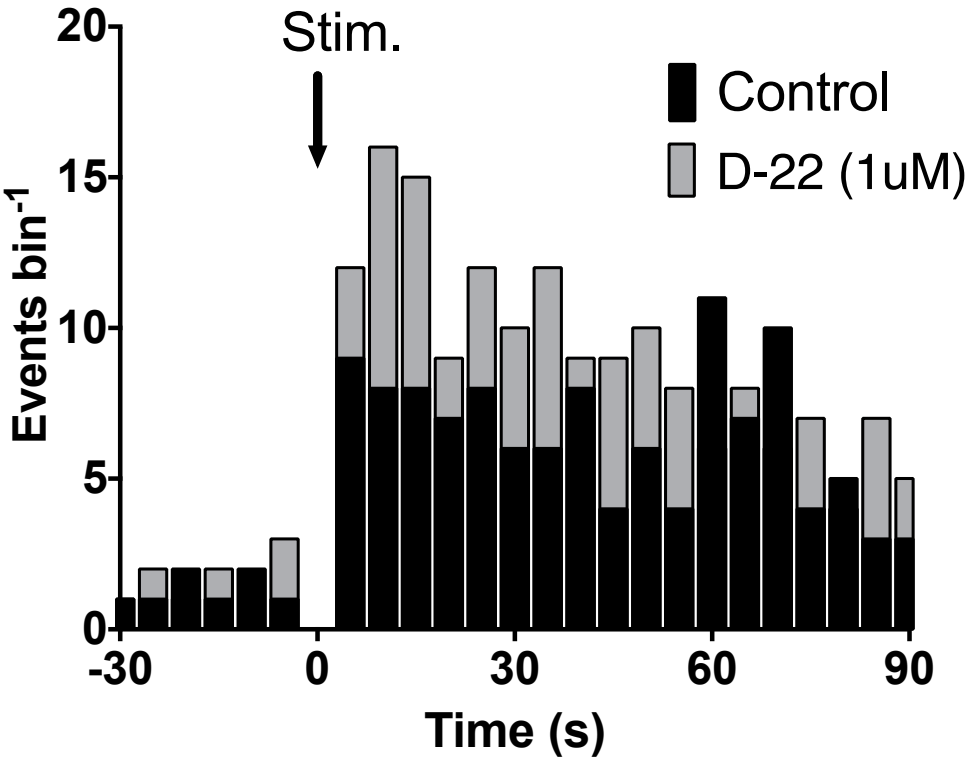
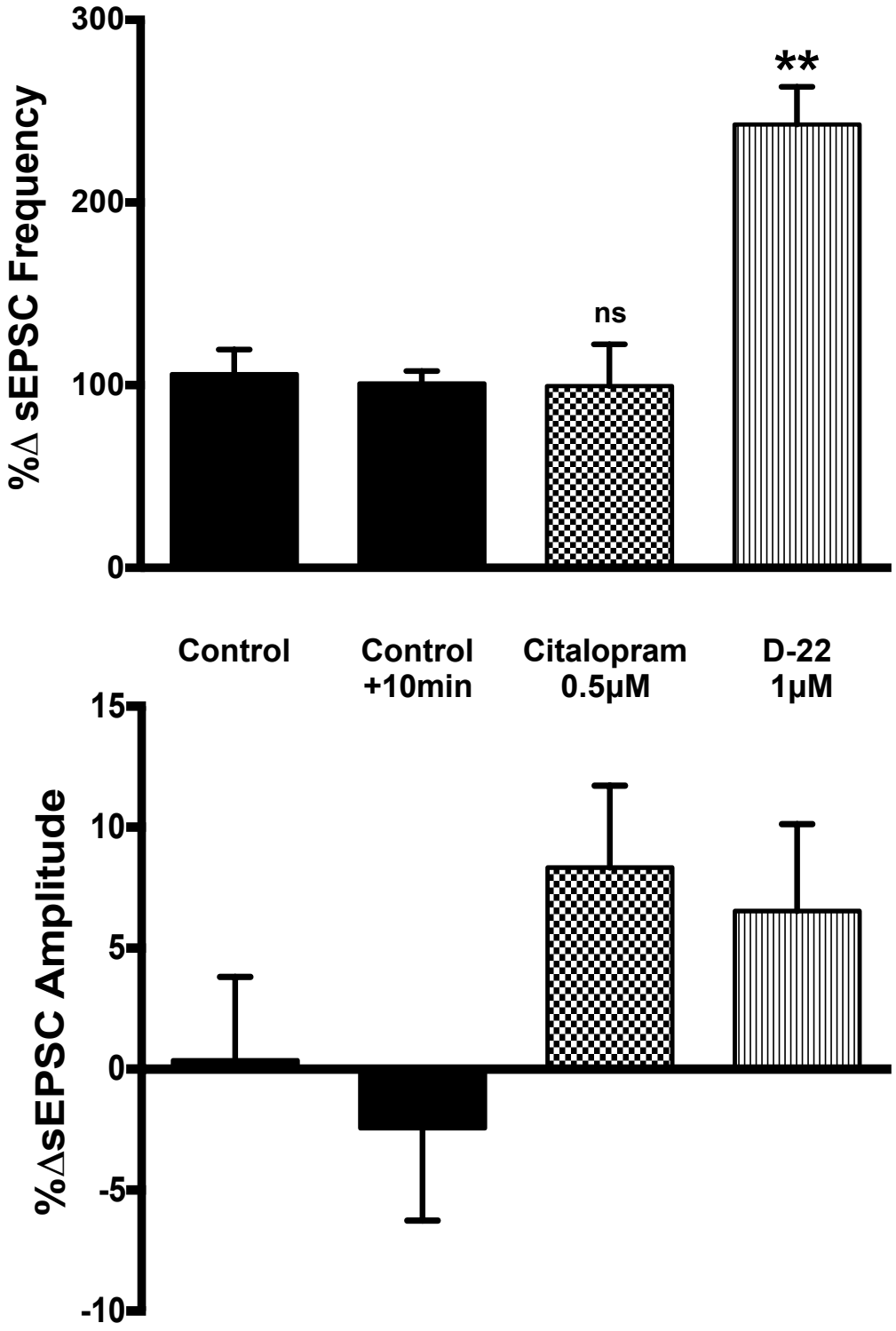


Figure 3.26 20Hz Train stimulation: Histogram

Frequency histogram of sEPSC frequency over time binned into 10s periods from a neuron recorded in the NTS before and after applying trains of 100 stimulation pulses to the solitary tract in the presence and absence of D-22 (1μM).

Figure 3.27 20Hz Train stimulation: Combined Data

Histogram of mean (±s.e.m) percentage changes over baseline in spontaneous EPSC (sEPSC) frequency and amplitude recorded in NTS cells measured over 60s post application of 100 stimulation pulses to the solitary tract. Control; aCSF + 25μM gabazine; n=9, Control =10min; aCSF + 25μM gabazine; n=9 10min after initial stimulation, citalopram (CIT; 0.5 μM, n=6) and D-22; 1μM (n=6). Compared to control with 1-Way ANOVA followed by Fisher's LSD test; $^{**}P<0.0$, ns; non-significant

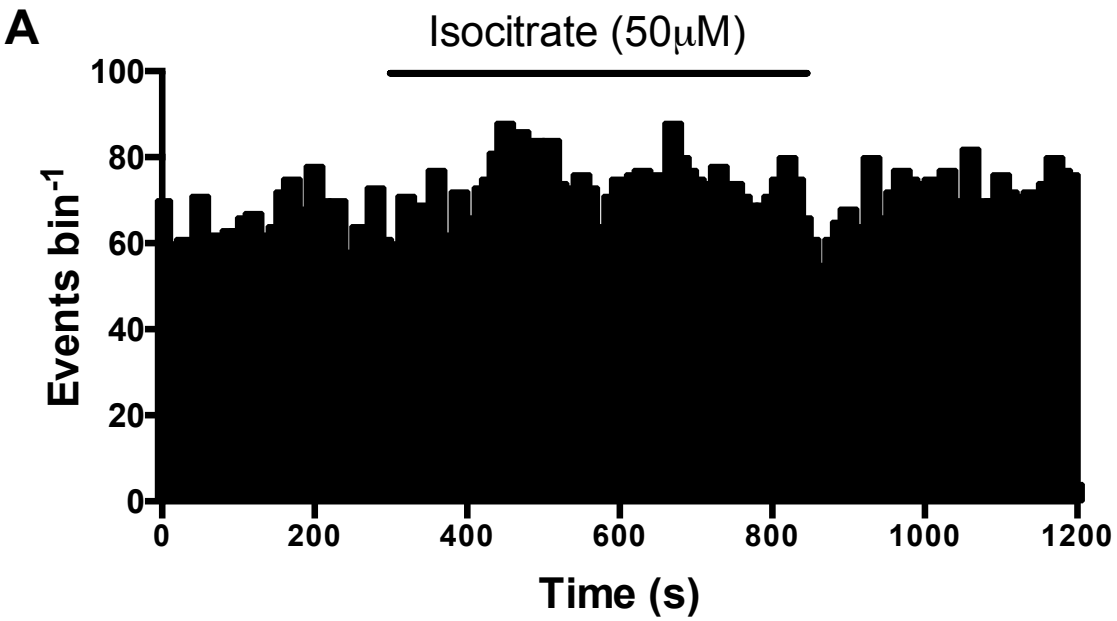

300
200
100
0
%Δ sEPSC Frequency
**
ns
Control
Control +10min
Citalopram 0.5µM
D-22 1µM
15
10
5
0
-5
-10
%ΔsEPSC Amplitude

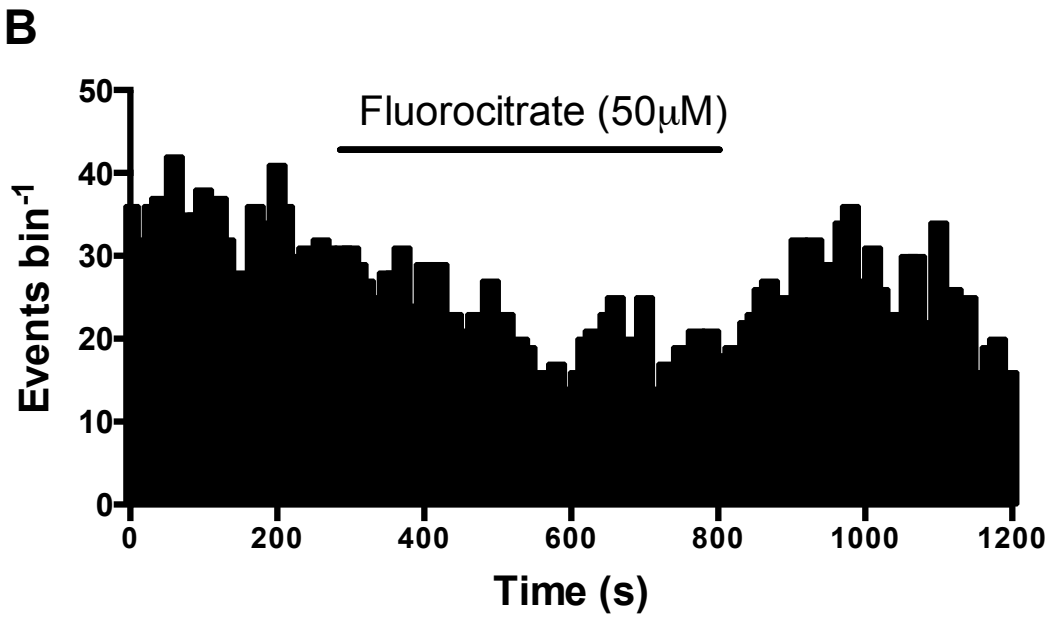
3.8. Effect of glial metabolic inhibitor fluorocitrate

Application of 50μM fluorocitrate decreased the frequency of spontaneous mEPSCs in NTS neurons tested (n=2; figure 2.8) by 41 ± 2%. On the other hand, the inactive analogue of fluorocitrate, isocitrate (50 μM) had no effect on mEPSC frequency (-0.2± 8.6%; n=3). In the presence fluorocitrate and isocitrate mEPSC amplitude was changed by -6.9 ± 1% and 0.6 ± 0.9%, respectively. Combined data is shown in figure 2.9.

Figure 3.28 Effect of isofluorocitrate on mEFPC frequency

Frequency histogram of mEPSC frequency over time binned into 10s periods from a neuron recorded in the NTS A) during the application of isocitrate (50μM) and B) during the application glial metabolic inhibitor fluorocitrate (50μM).


A
Isocitrate (50μM)
Events bin-1
100
80
60
40
20
0
0
200
400
600
800
1000
1200
Time (s)


B
Fluorocitrate (50μM)
Events bin-1
50
40
30
20
10
0
0
200
400
600
800
1000
1200
Time (s)

3.9. Discussion

3.9.1. Main findings

The present studies focused on the modulation of glutamate release by 5-HT_3 and 5-HT_{1A} receptors examined in the brainstem slice preparation. The data indicates that 5-HT_3 receptors are tonically activated in this preparation and modulate the frequency of glutamate release from a presynaptic location. Attempts to augment this spontaneous release of 5-HT with re-uptake inhibitors caused a paradoxical decrease in mEPSC frequency. This was found to be caused by activation of presynaptic 5-HT_{1A} receptors. Indeed, 5-HT itself has a biphasic dose response curve when applied exogenously, with low doses causing inhibition *via* activation of the 5-HT_{1A} receptor. Higher doses of 5-HT cause excitation *via* activation of the 5-HT_3 receptor.

The 5-HT transporter re-uptake inhibitor failed to increase the frequency of spontaneous EPSCs induced by stimulating the solitary tract with a train of pulses, whereas the OCT_3/PMAT inhibitor D-22 caused an increase in this release. This indicates that OCT_3/PMAT are responsible for the removal of 5-HT from the extracellular space when concentrations are high subsequent to stimulation.

Finally, preliminary data indicates that glia cells present in the NTS are a source of some the spontaneously released glutamate seen in this preparation.

3.9.2. Spontaneous release

The ability of the selective 5-HT_3 antagonist granisetron (Sanger & Nelson, 1989) to reduce spontaneous glutamatergic mEPSCs confirms the observations of Wan and Browning (2008) that there is spontaneous release of 5-HT within the NTS slice. This acts *via* 5-HT_3 receptors on vagal afferent terminals to release glutamate in the NTS in rat brainstem slice preparations treated with TTX and gabazine. In this respect, activation of 5-HT_3 receptors in the present experiments with the $5HT_3$

agonist phenylbiguanide causes the expected increases in glutamatergic mEPSCs. This effect is attenuated by granisetron. This was a particularly powerful effect causing, in some cases, an increase of >2000% in mEPSC frequency and was dose related.

In contrast 5-HT caused a dose related biphasic effect on mEPSCs frequency. At low doses inhibition was observed while at high doses there was excitation. This excitation was, as seen with PBG, associated with very large increases mEPSCs frequency. The inhibition by low doses was found to be blocked by pretreatment with the 5-HT_{1A} antagonist WAY-100635 (Forster *et al.*, 1995), while the high dose excitation could be reversed to inhibition in the presence of the 5-HT_3 antagonist granisetron. WAY-100635 alone had no effect on mEPSCs, indicating that this spontaneously released 5-HT is not able to activate the 5-HT_{1A} receptor. The affinity for 5-HT at 5-HT_{1A} receptors is approximately 20 times higher than at 5-HT_3 receptors; pK_i 8.2 at the rat 5-HT_{1A} receptor (Watson *et al.*, 2000) compared to 6.8 at the rat 5-HT_3 receptor (Kilpatrick *et al.*, 1989). Comparing the dose of 5-HT that causes inhibition (0.3 μM) to that which cause excitation (1μM), in the present experiments, would suggest that this selectivity would be lower than that seen in an *in vitro* expression system, possibly as low as 3 times. This will also explain why the low concentrations of applied 5-HT activated 5-HT_{1A} receptors causing inhibition of mEPSCs before activating 5-HT_3 receptors to produce excitation. However, as spontaneously released 5-HT causes an increase in mEPSCs frequency, this would imply that the sites releasing 5-HT are nearer to the $5HT_3$ than the 5-HT_{1A} receptors. Further, the size of the 5-HT_3-mediated effect on glutamatergic mEPSCs is consistent with that observed *in vivo* (Wang *et al.*, 1997, Ramage & Mifflin, 1998, Jeggo *et al.*, 2005) supporting the view these receptors are important in visceral afferent processing in the NTS.

Blockade of 5-HT reuptake *via* either the high-affinity low-capacity transporter (5-HT transporter or SERT) with citalopram or the low-affinity high-capacity transporters (OCT3/PMAT) with decynium-22 caused a decrease in mEPSCs frequency rather than the expected increase. This decrease was prevented by pretreatment WAY-100635, indicating that blockade of 5-HT removal from the extracellular space allows the spontaneously released 5-HT to activate 5-HT_{1A}

receptors. Further, this now overrides the background activation of $5HT_3$ receptors. Surprisingly, blockade of either uptake system, even with larger doses of the uptake inhibitors, in the presence of WAY-100635 failed to produce a 5-HT_3-mediated increase in glutamatergic mEPSCs. Although with the high dose of decynium-22 there was a small but observable increase in mEPSCs frequency. It should be noted that this was a very small effect when compared to that caused by the high dose of 5-HT added to the slice; a 57 ± 39% increase compared to a 2462 ± 543% increase in mEPSCs frequency. This implies that these uptake transporters do not play a major role in the regulation of the low levels of extracellular 5HT caused by its spontaneous release in the vicinity of these $5HT_3$ receptors, but do limit the availability of 5-HT at 5-HT_{1A} receptors. This is particularly intriguing because the 5-HT_{1A} receptor effect is predominant when a low concentration of 5-HT is added to the slice. Again, this supports the view that the 5-HT_3 receptor is in very close proximity to this spontaneous 5-HT release site and local extracellular 5-HT concentration in this area is not regulated, or is poorly regulated, by uptake transporters. This data also indicates that both types of uptake blockade cause a similar rise in the background extracellular concentration of 5HT, at least when 5-HT is spontaneously released.

Further, the failure of corticosterone, a OCT3 inhibitor (Engel & Wang, 2005), to have any effect on mEPSCs frequency suggests that it is PMAT rather than OCT3 that is primarily involved in the uptake of 5-HT. This view is also supported by *in vivo* data seen in chapter 4 showing corticosterone having no effect on the uptake of exogenous 5-HT applied to the NTS.

The ability of 5-HT reuptake blockade to cause activation of 5-HT_{1A} receptors is reminiscent of one of the explanations for the delay in onset of the therapeutic affect of 5-HT uptake inhibitors in the treatment of depression. That is, although 5-HT uptake is blocked immediately, the activation of 5-HT_{1A} receptors causes an initial reduction in the overall release of 5-HT in the cortex until the 5-HT_{1A} receptor desensitizes (see Artigas *et al*., 1996). However, there no evidence in the NTS that 5-HT_{1A} receptors are acting, as they do in the dorsal raphé, as somatodendritic autoreceptors. Therefore, NTS 5-HT_{1A} receptors should be considered postsynaptic 5-HT_{1A} receptors, presumably similar to those that have been well described in the

hippocampus (see Riad *et al*., 2000, Polter & Li, 2010). Further the present observation that activation of 5-HT_{1A} receptors failed to decrease the amplitude of the mEPSCs indicates that these receptors are presynaptic to the neurons from which recordings were made. Although 5-HT_3 receptors have been shown to be predominantly located on vagal afferents terminals, there is no experimental evidence that $5HT_{1A}$ receptors are also found on these terminals. Nonetheless, in the analogous primary afferent system in the spinal cord, a small proportion of the $5HT_{1A}$ receptors have been found on primary afferent fibers along with 5-HT_3 receptors. (Laporte *et al*., 1995) Thus, by analogy, there may be a few on vagal afferents terminals. It should be noted that the NTS has a high density of 5–HT_{1A} receptors (Pompeiano *et al*., 1992), although *in vivo* experiments have so far failed to determine their role in the regulation of vagal afferent excitation of NTS neurons (Oskutyte *et al*., 2009).

Fluorocitrate is shown to reduce the glutamatergic mEPSC frequency recorded in NTS cells. Fluorocitrate, selectively taken up by astrocytes, suppresses the citric acid cycle and reduces glutamine production in microglia and astrocytes leading to a depravation of energy in these cells (Hassel *et al*., 1992, Fonnum *et al*., 1997). The turnover of neurotransmitters within a cell is particularly energy intensive so release of transmitter would be prevented if energy production were to be halted. This data indicates that approximately 40% of the recorded mEPSC result from glutamate released from glia. Indeed, glutamate has been previously identified as a gliotransmitter (Halassa *et al*., 2007). To confirm the specificity of fluorocitrate, isocitrate, an inactive isomer, was administered but had no effect on mEPSC frequency. This, therefore, reduces the likelihood of the observation being a result of off-target effects.

3.9.3. Evoked release

The present observation that the amplitudes of the evoked (by solitary tract stimulation) EPSC is reduced and the pulse paired ratio is increased in the presence of granisetron is again similar to that previously observed with the 5-HT_3 antagonist

ondansetron (Wan & Browning, 2008) and can be interpreted (Mennerick & Zorumski, 1995, see Zucker & Regehr, 2002) to indicate that again these 5-HT_3 receptors are presynaptic to the recording site and that vagal afferent excitation causes the release of 5-HT in the NTS. Both doses of citalopram failed to affect either amplitude of evoked EPSC or the paired pulse ratio. This supports the view that the high-affinity low-capacity transporter (the 5-HT transporter) is not involved in the regulation of this glutamate-releasing $5HT_3$ receptor pathway, even when driven. However, the ability of decynium-22 to increase the amplitude of evoked EPSC and decrease the paired pulse interval does indicate that OCT3/PMAT plays a role in the regulation of the extracellular concentration of 5HT, when it is increased by vagal afferent stimulation but not the background concentration of 5-HT caused by its spontaneous release. This again was observed for a train of stimuli at 20Hz. Vagal afferent stimulation at this frequency causes a consistent detectable 5-HT release *in vivo* in the NTS (Hosford *et al*., 2011, see thesis chapter 5). The combined data supports the view that OCT3/PMAT but not the 5-HT transporter is involved in the regulation of the increase in the extracellular concentration of 5-HT in response to vagal afferent stimulation. However, the failure to see an effect with citalopram is surprising, although consistent with *in vivo* experiments (Hosford *et al*., 2012). The 5-HT transporter appears to be only involved in the regulation of extracellular 5-HT levels in this 5-HT_{1A} pathway. This is again consistent with the *in vivo* data that 5-HT_{1A} receptors are not involved in the regulation of vagal afferent release of 5-HT in the NTS (Oskutyte *et al*., 2009). Even if they were involved, the failure to detect a 5-HT_{1A}-mediated effect in the presence of either uptake inhibitor would not be surprising because a large increase in the extracellular level of 5-HT, as shown by adding high doses 5-HT to the slice, would easily override the 5-HT_{1A}-mediated inhibitor effect on spontaneous glutamate release. The site(s) at which vagal afferent activation causes this release of 5-HT remains to be determined. It could be directly from these afferents themselves, possibly a subpopulation, and/or from 5-HT-containing nerve terminals originating from the medullary raphe, of which there is significant histological evidence (see INTRODUCTION 1.3). However, the site must be in close juxtaposition to vagal afferent terminals.

3.9.4. Conclusion

The present data demonstrates that the low-affinity, high-capacity monoamine uptake transporter (PMAT) and not the high-affinity, low-capacity transporter (5-HT transporter) is involved in the regulation of the rise in extracellular concentration of 5-HT in the NTS in response vagal afferent stimulation. This rise in 5-HT activates 5-HT_3 receptors, which in turn cause the release of glutamate, confirming *in vivo* observations (Jeggo *et al*., 2005). The data from Wan and Browning (2008) indicates that this 5-HT-dependent glutamate release comes from vagal afferent terminals. However, it has been suggested that glia may also provide either an additional or alternate source (Jeggo *et al*., 2005). Indeed, data from the present study showing application of the glia-specific metabolic inhibitor fluorocitrate reduces mEPSC frequency supports this view. Nevertheless, when studying spontaneous release of 5-HT within the NTS in brainstem slices, both types of uptake blockade were equally as effective in regulating the extracellular concentration and preventing the 5-HT concentration rising high enough to activate 5-HT_{1A} receptors. Such blockade, surprisingly, had little effect on 5-HT_3 receptor-mediated release of glutamate and this was interpreted to indicate that the 5-HT release site is close to these receptors. However the role of these $5HT_{1A}$ receptors in NTS neuronal regulation remains to be determined, although they are protected from activation by the spontaneous released 5-HT by both uptake systems and are preferentially activated by low concentrations of 5-HT, at least when compared to 5-HT_3 receptors. The physiological reason for this remains to be determined. As there is yet no evidence that these $5HT_{1A}$ receptors are involved in the vagal afferent activation of NTS neurons. This suggests that they may be involved other NTS functions not related to visceral afferent processing. The data does indicate that when dealing with the evoked release of 5-HT the low-affinity, high-capacity transporter, probably PMAT, is more important than the 5-HT transporter for regulating the changes in extracellular cellular levels of 5-HT caused by stimulated release.

3.9.5. Future experiments

The source of the 5-HT release has yet to been determined. There are a number of possible sources for this release and the relative contributions of each source require further investigation. This can be achieved by selective removal of 5-HT from two sources:

Firstly, vagal afferent terminals are known to contain 5-HT (Nosjean *et al.*, 1990, Sykes *et al.*, 1994) and experiments by Wan and Browning (2008) have shown that differentiation by unilateral vagotomy abolishes the inhibitory effect of 5-HT_3 antagonists on mEPSC frequency in the NTS. However, this could be the result of either the removal of vagal afferent terminal expressing 5-HT_3 or the removal of terminals releasing 5-HT, or both. 5,7-Dihydroxytryptamine (5,7-DHT) has been used to selectively destroy the 5-HT-containing pathway from the nodose ganglion to the NTS (Orer *et al.*, 1991). The experimental approach would involve unilateral microinjection of 5,7-DHT into the nodose ganglion to remove the 5-HT-containing pathway without destroying the main glutamatergic input into the NTS. Experiments can be repeated as previously described on both sides of the NTS in a single brainstem slice in order to compare differences between the 5-HT depleted side and the control side. If 5-HT_3 antagonists were less effective in the depleted side then this would support the view that 5-HT is release from afferent terminals within the NTS.

Secondly, 5-HT input from medullary raphe nuclei into the NTS is also a likely source of 5-HT seen released within the present experiments. Within the brainstem slice there has been some suggestions that the horizontal slice configuration remove input originating from medullary nuclei and thus 5-HT release (Cui *et al.*, 2012). However, in this configuration the 5-HT-containing terminals will still be present in the NTS and may be the source of some of the 5-HT release seen in the horizontal slice used for some of the present experiments. In this case it would be necessary to target the medullary nuclei themselves in order to remove the input to the NTS. Microinjection of 5,7-DHT into the medullary raphe nuclei would be a possible experimental approach.

4. ASSESSMENT OF MONOAMINE UPTAKE SYSTEMS IN THE NTS

4.1. Introduction

Biogenic amine reuptake inhibitors are extremely useful experimental tools are routinely used to aid in the identification of electroactive species measured by *in vivo* electrochemistry (see Phillips & Wightman, 2003). However, as with any pharmacological agent, correct dosing to maintain selectivity is paramount. Unfortunately, much of the current data regarding selectivity of these compounds relies on receptor binding studies conducted in expression systems. Further, meta-analysis (see Stanford, 1996) of the earlier literature shows that compounds previously described as 'serotonin-selective reuptake inhibitors' are mixed noradrenaline and 5-HT re-uptake inhibitors, to a greater or lesser extent (see INTRODUCTION 1.5).

Studies into the acute effect of re-uptake inhibitors *in vivo* often use doses in the rage of 5-10mg kg^{-1} and almost always administer these compounds i.p. (for example Hashemi *et al.*, 2009, Karlsson *et al.*, 2013, Ortega *et al.*, 2013). When administering i.p higher doses are used due lower bioavailability than with i.v administration and plasma concentration is less predictable. However, but due to the ease of application of this method it is often used preferentially. Consequently, there is little data regarding dosing of these compounds using the i.v. route. It would seem that, given the affinity at the binding site is in the low nM range for most re-uptake inhibitors (Tatsumi *et al.*, 1997), doses of 10mg kg^{-1} would ensure maximum occupancy. Still, the selectivity and relative potency of these compounds is largely unknown *in vivo*. It is, therefore, difficult to select an appropriate dose when using these pharmacological tools and selectivity is extremely important when validating electrochemical signals. The present study was designed to address the paucity of data in this area and gain an insight into the selectivity and effective doses of re-uptake inhibitors in order for the tools to be more confidently used when identification of electroactive species.

4.2. Specific Aims

- Determine the uptake kinetics of exogenously applied 5-HT, noradrenaline and dopamine in the NTS by voltammetry
- Evaluate the effects of commonly used selective re-uptake inhibitors on these parameters
- Evaluate the effects of inhibitors of alternative re-uptake systems OCT_3 and PMAT on these parameters
- Compare selectivity ratios of the drugs currently available

4.3. Effect of re-uptake inhibitors on 5-HT clearance

Effect of drugs targeting SERT on decay time

The SERT inhibitor citalopram (1mg kg^{-1}, i.v; n=7, figure 4.1A) caused a significant increase in the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}) when compared to control. In the presence of citalopram T_{80} increased by 36.9 ± 4.6% over pre-drug control. At the same dose fluoxetine (1mg kg^{-1}, i.v; n=4, figure 4.1B) caused a change in T_{80} of -0.7 ± 5.8% over pre-drug controls. This was not significant. These values were compared for significance with a time-matched vehicle control that only changed by -2.6 ± 2.6% (n=6). Combined data is shown in figure 4.3.

Effect of drugs targeting the noradrenaline transporter on decay time

Reboxetine (1mg kg^{-1}, i.v; n=6, figure 4.2A), a noradrenaline selective re-uptake inhibitor, caused an increase of 12.5 ± 3.2% in T_{80} over pre-vehicle controls. This was significantly different from time-matched vehicle control. On the other hand, desipramine (1mg kg^{-1}, i.v; n=4, figure 4.2B), a tri-cyclic anti depressant, caused a change of 4.7 ± 5.7% in T_{80} over pre-vehicle controls. This was not significantly different from time-matched vehicle control. Combined data is shown in figure 4.3.

Figure 4.1 Representative experimental traces of 5-HT clearance: Effect of fluoxetine and citalopram

A) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of citalopram (1mg kg^{-1}, i.v).

B) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of fluoxetine (1mg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).

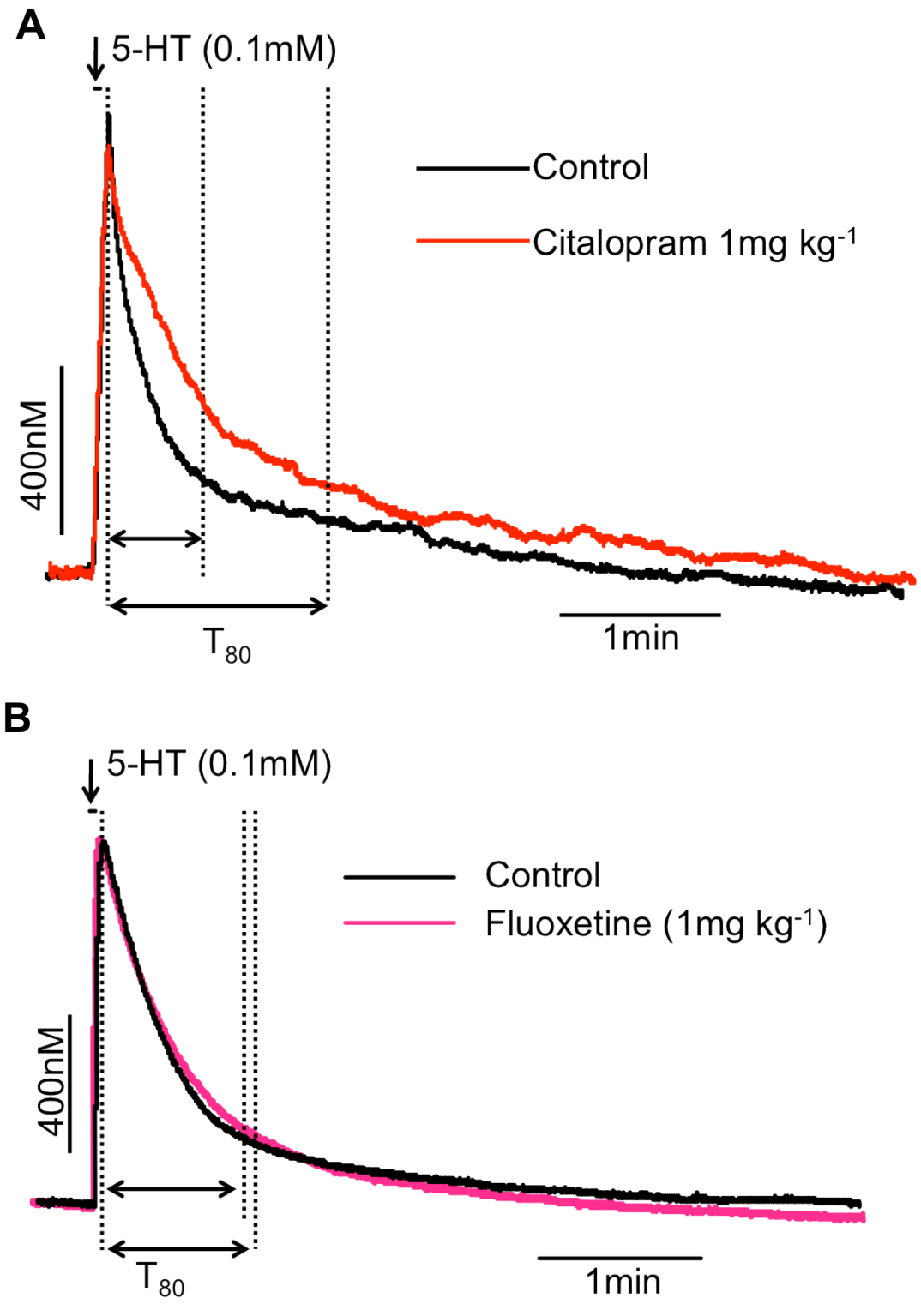
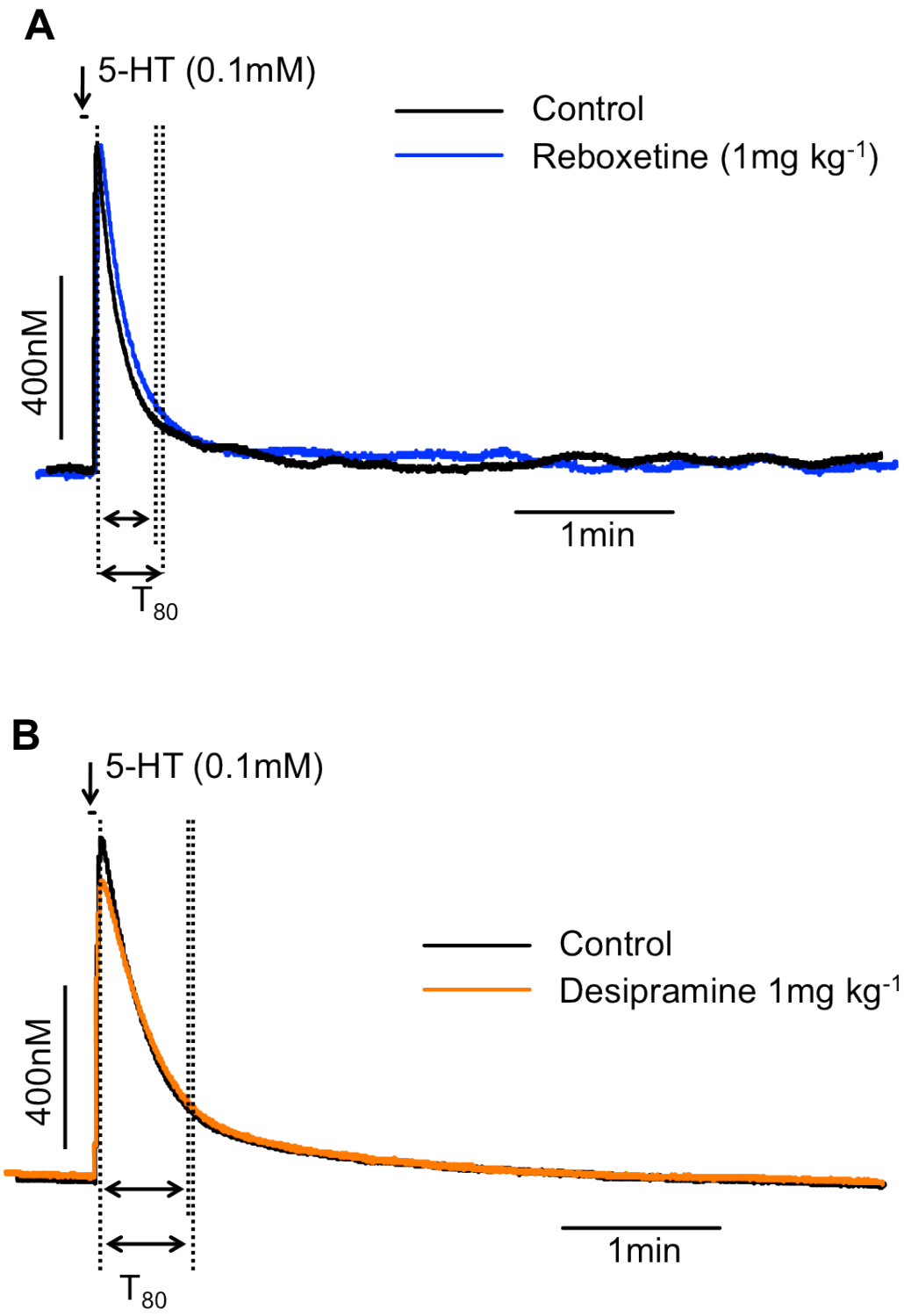

A
5-HT (0.1mM)
Control
Citalopram 1mg kg-1
400nM
T80
1min
B
5-HT (0.1mM)
Control
Fluoxetine (1mg kg-1)
400nM
T80
1min

Figure 4.2 Representative experimental traces of 5-HT clearance: Effect of reboxetine and desipramine

A) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of reboxetine (1mg kg^{-1}, i.v).

B) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of desipramine (1mg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).


A
5-HT (0.1mM)
Control
Reboxetine (1mg kg-1)
400nM
1min
T80
B
5-HT (0.1mM)
Control
Desipramine 1mg kg-1
400nM
1min
T80

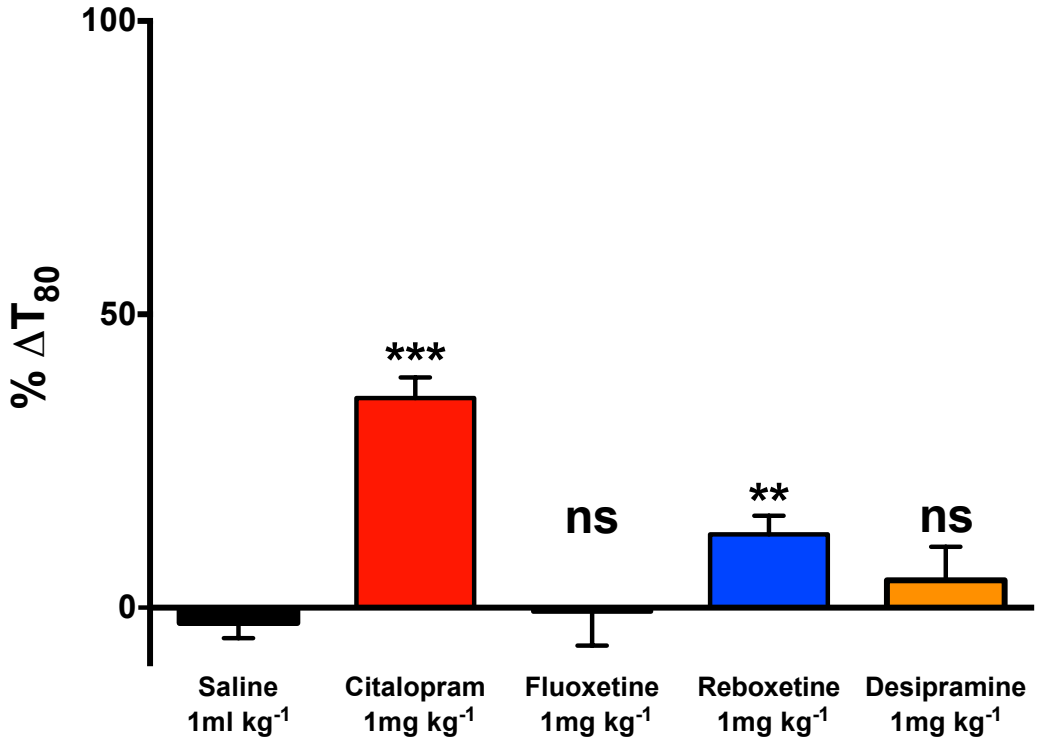


Figure 4.3 Effect of re-uptake inhibitors on noradrenaline clearance: Combined data

Histograms of mean (±s.e.m) percentage changes over pre-drug control in the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}). Electrochemical signal was produced by microinjection of 0.1μM of 5-HT into the NTS. The decay time after drug or vehicle application of three microinjections of 5-HT was compared with microinjections three pre-drug or vehicle. Saline vehicle control; n=6, Citalopram; 1mg kg^{-1}; n=7, Fluoxetine; 1mg kg^{-1}; n=4, Reboxetine; 1mg kg^{-1}; n=4 and Desipramine; 1mg kg^{-1}; n=4.

** P<0.01,*** P<0.001, ns; non-significant; 1-Way ANOVA; compared to saline with Fisher's LSD test.

Effect of drugs targeting OCT_3 on decay time

In the presence of corticosterone (1mg kg^{-1}, i.v, n=3, figure 4.4A), an inhibitor of OCT_3, the T_{80} only changed by 3.4 ± 1.8% over pre-drug controls. Higher doses of 3mg (n=1) and 10mg kg^{-1} (n=5) also had a similar effect; the T_{80} changed by 4.9% and 3.3 ± 2.4%, respectively over pre-drug controls. This was not significantly different from time-matched vehicle controls at any dose. Combined data is shown in figure 4.5.

Effect of drugs targeting OCT_3/PMAT on decay time

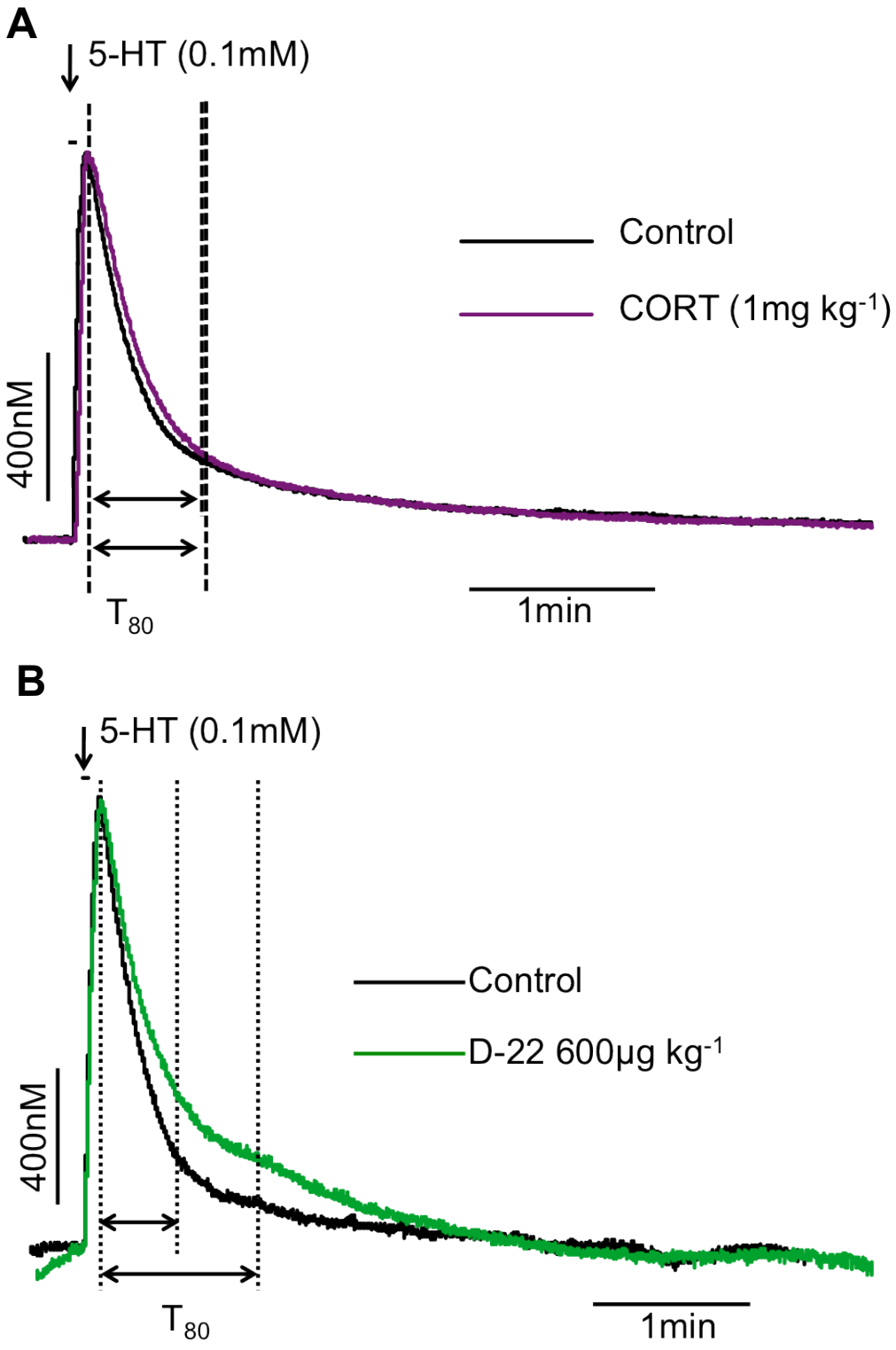
In the presence of decynium-22 (D-22; 600µg kg^{-1}, i.v, n=5, figure 4.4B), an inhibitor of OCT_3/PMAT, the T_{80} increased by 76.0 ± 12.8% over pre-drug controls. This was significantly different from time-matched vehicle control after which T_{80} only changed by 1.7 ± 1.6% (n=8). Combined data is shown in figure 4.5.

Figure 4.4 Representative experimental traces of 5-HT clearance: Effect of corticosterone and decynium-22

A) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of corticosterone (CORT; 1mg kg^{-1}, i.v).

B) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of 5-HT into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of decynium-22 (D-22; 600µg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).


A
5-HT (0.1mM)
Control
CORT (1mg kg-1)
400nM
T80
1min
B
5-HT (0.1mM)
Control
D-22 600µg kg-1
400nM
T80
1min

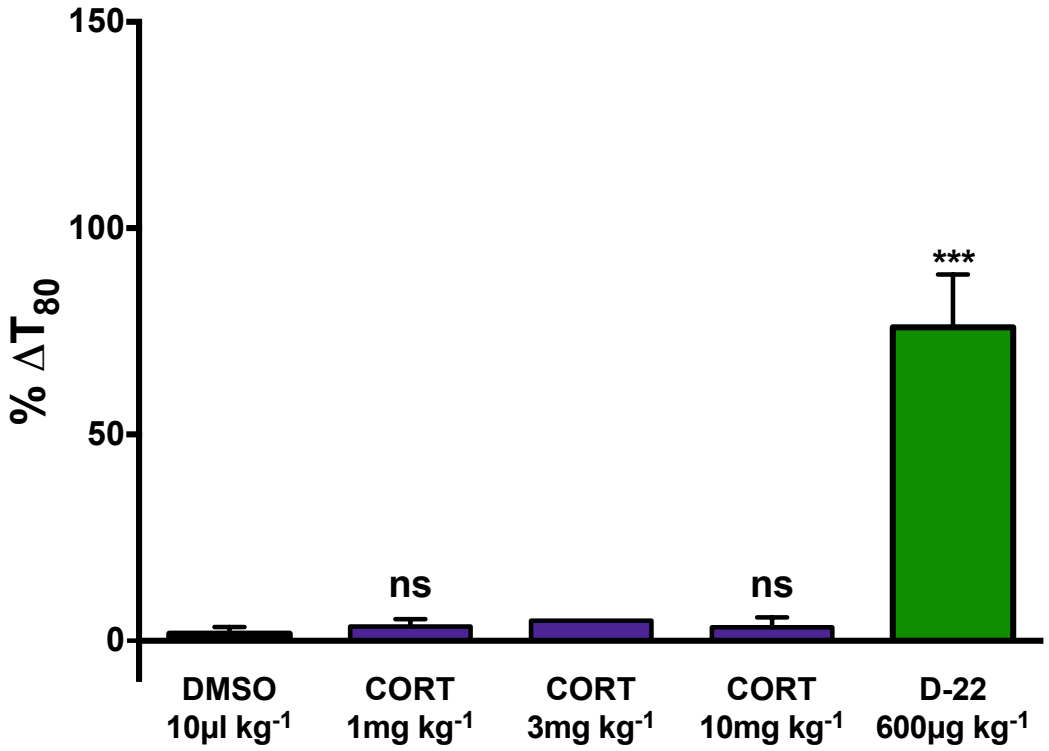


Figure 4.5 Effect of OCT3/PMAT blockade on 5-HT clearance: Combined data

Histograms of mean (±s.e.m) percentage changes over pre-drug control in the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}). Electrochemical signal was produced by microinjection of 0.1µM of 5-HT into the NTS. The decay time after drug or vehicle application of three microinjections of 5-HT was compared with microinjections three pre-drug or vehicle. DMSO vehicle control; n=8, corticosterone 1,3 and 10mg kg^{-1} (CORT 1,3 and 10mg kg^{-1}; n= 3, 1 and 5) and decynium-22 (D-22; 600µg kg^{-1}, i.v, n=5).

** P<0.01, ns; non-significant; 1-Way ANOVA; compared to saline with Fisher's LSD test.

4.4. Effect of re-uptake inhibitors on NA clearance

In the presence of reboxetine (1mg kg^{-1}, i.v; n=5, figure 4.6A), a noradrenaline selective re-uptake inhibitor, the T_{80} increased by 58.0 ± 4.2% in T_{80} over pre-drug controls. This was significantly different from time-matched vehicle control after which T_{80} only changed by 0.64 ± 1.2% (n=5).

In the presence of desipramine (1mg kg^{-1}, i.v; n=5, figure 4.6B), a tri-cyclic antidepressant, the T_{80} increased by 82.6 ± 16.9% in T_{80} over pre-drug controls. This was also significantly different from time-matched vehicle control.

In the presence of citalopram (1mg kg^{-1}, i.v; n=4, figure 4.7), the selective 5-HT re-uptake inhibitor, the T_{80} only changed by 2.3 ± 3.3% in T_{80} over pre-drug controls. This was not significantly different from time-matched vehicle control.

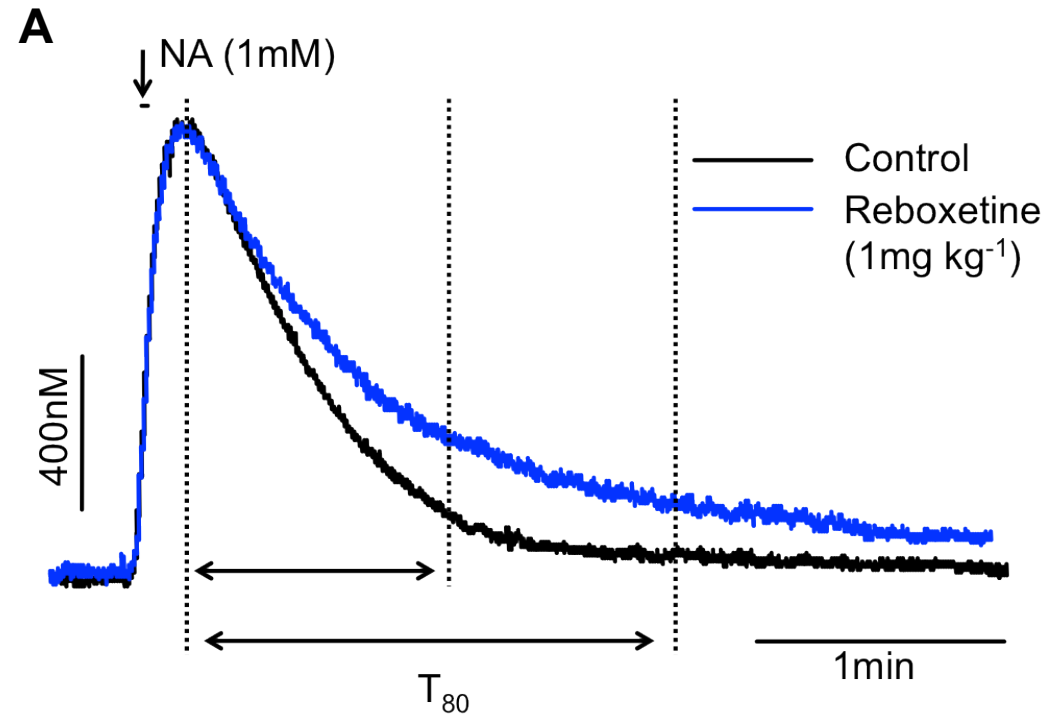
Combined data is shown in figure 4.8.

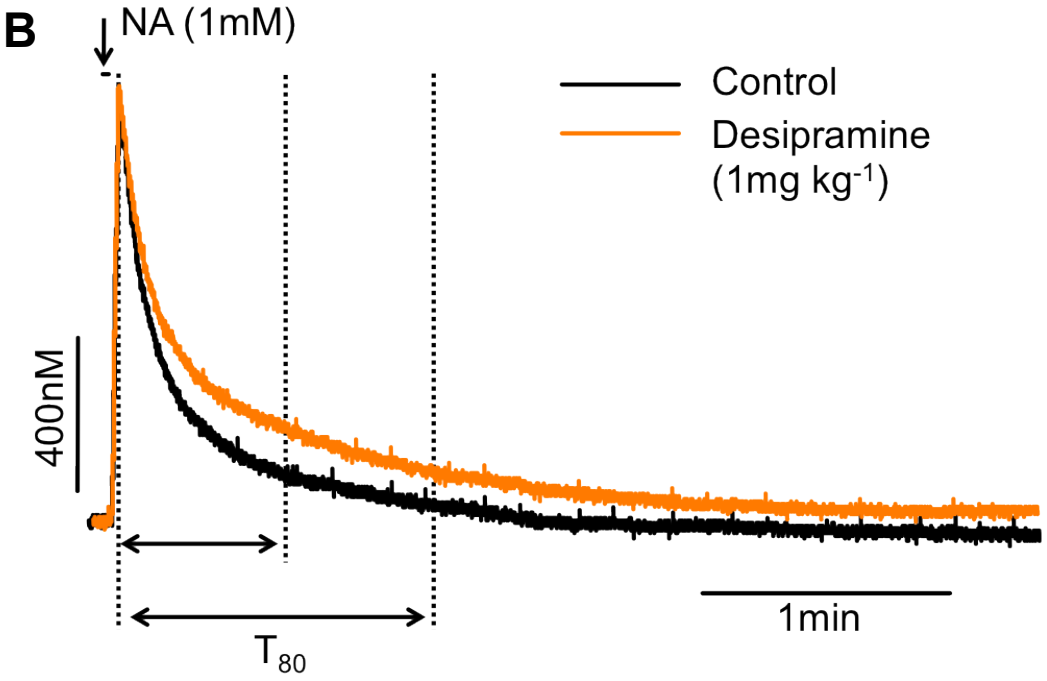
Figure 4.6 Representative experimental traces of noradrenaline clearance: Effect of corticosterone and decynium-22

A) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of noradrenaline (NA) into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of reboxetine (1mg kg^{-1}, i.v).

B) Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of noradrenaline (NA) into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of desipramine (1mg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).


A
NA (1mM)
Control
Reboxetine
(1mg kg-1)
400nM
T80
1min


B
NA (1mM)
Control
Desipramine
(1mg kg-1)
400nM
T80
1min

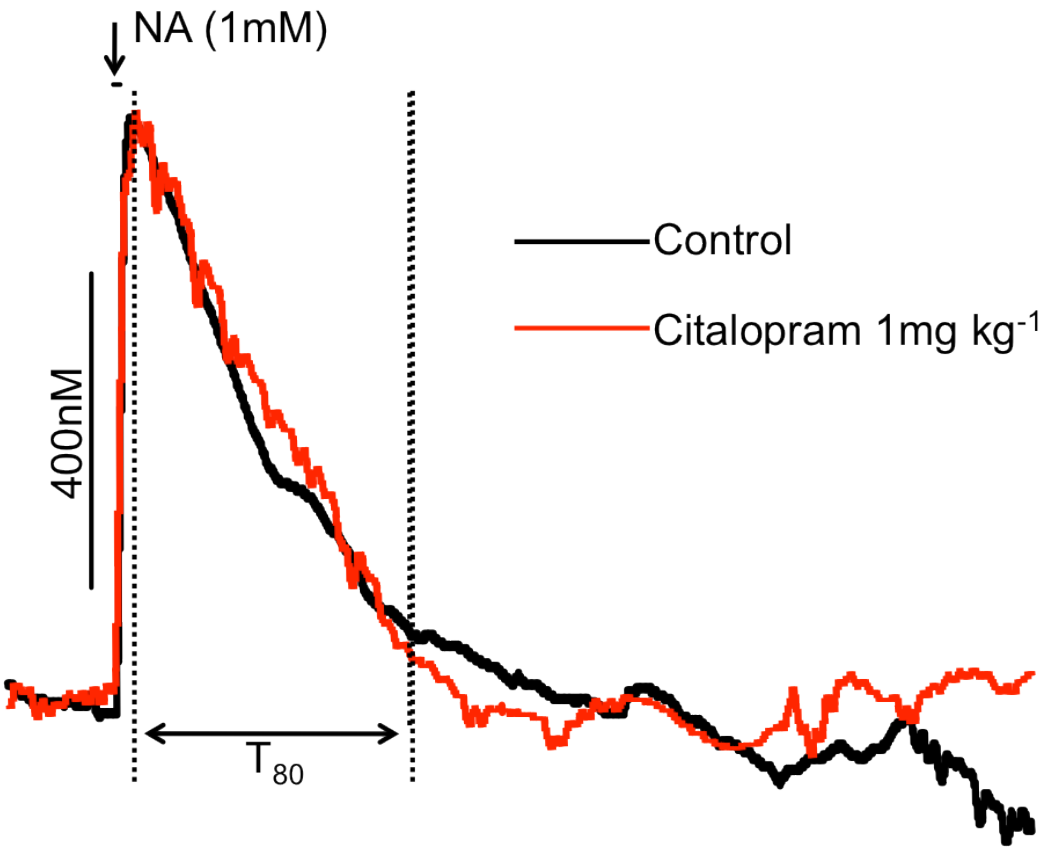


Figure 4.7 Representative experimental trace of noradrenaline clearance: Effect of citalopram

Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of noradrenaline (NA) into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of citalopram (1mg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).

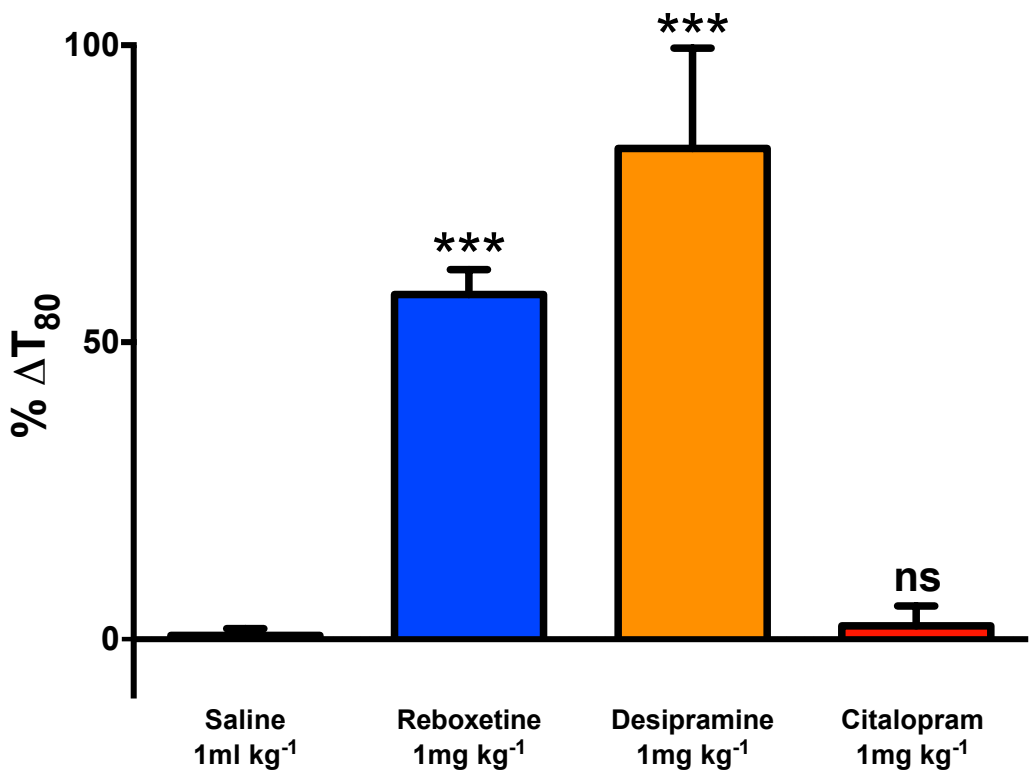


Figure 4.8 Effect of re-uptake inhibitors on 5-HT clearance: Combined data

Histograms of mean (±s.e.m) percentage changes over pre-drug control in the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}). Electrochemical signal was produced by microinjection of 1μM of noradrenaline (NA) into the NTS. The decay time after drug or vehicle application of three microinjections of 5-HT was compared with microinjections three pre-drug or vehicle. Saline vehicle control; n=5, reboxetine; 1mg kg^{-1}; n=5, citalopram; 1mg kg^{-1}; n=4 and desipramine; 1mg kg^{-1}; n=5

*** P<0.001, ns; non-significant; 1-Way ANOVA; compared to saline with Fisher's LSD test.

4.5. Effect of re-uptake inhibitors on dopamine clearance

Effect of drugs targeting DAT on decay time

After application of the DAT inhibitor GBR 12909 (1mg kg^{-1}, i.v; n=3, figure 4.9) the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}) only changed by -1.0 ± 1.8% compared to pre-drug control. This was not significantly different to a time-matched vehicle control that changed by -11.8 ± 5.9% (n=3, figure 4.10).

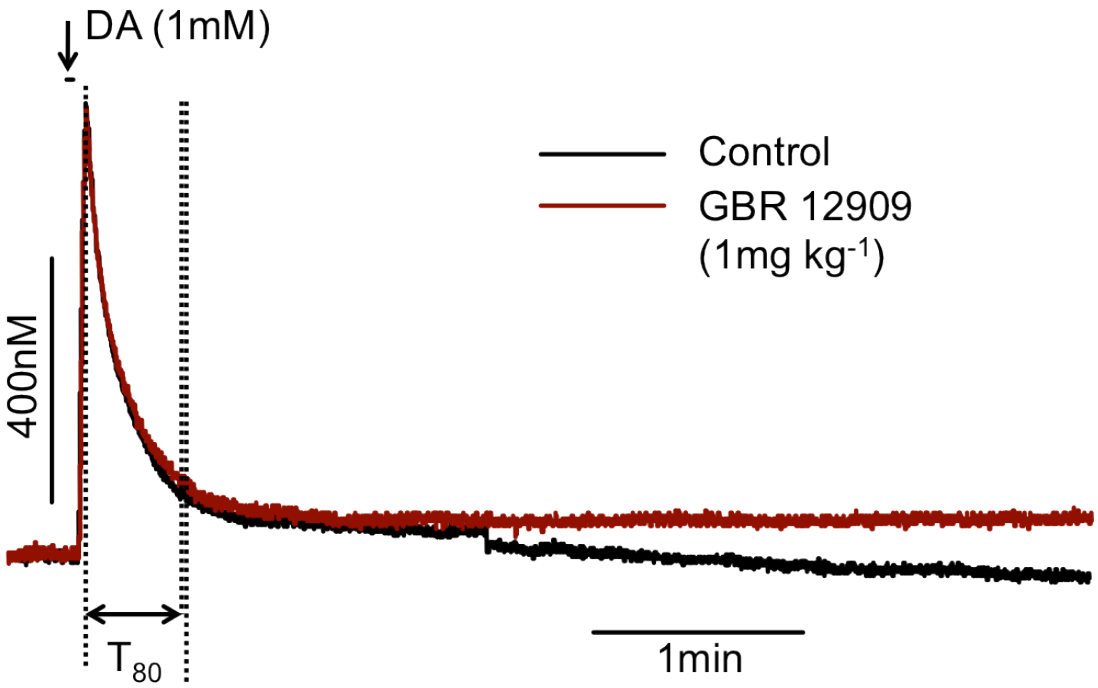


Figure 4.9 Representative experimental trace of dopamine clearance: Effect of GBR 12909

Representative oxidation currents converted to relative concentration (nM), produced by pressure ejection of dopamine (DA; 1mM) into the NTS. Pre-drug control (black) is overlaid with a microinjection taken in the presence of GBR 12909 (1mg kg^{-1}, i.v).

Vertical dashed lines indicate the time taken for the signal to decay to 80% of the peak concentration (T_{80}).

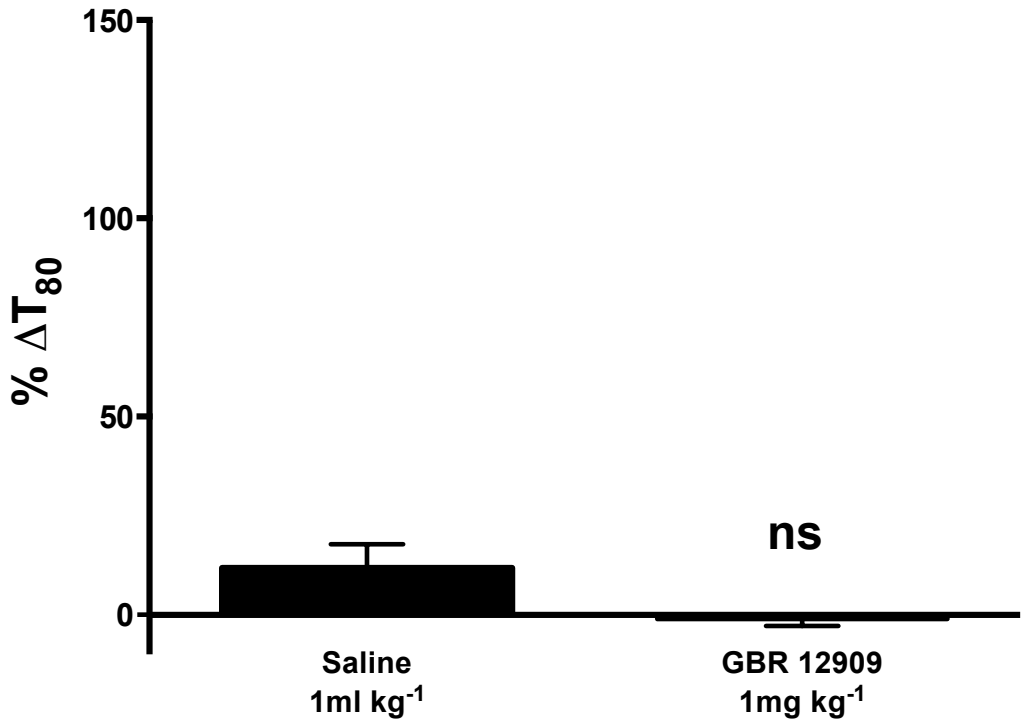


Figure 4.10 Effect of re-uptake inhibitors on dopamine clearance: Combined data

Histograms of mean (±s.e.m) percentage changes over pre-drug control in the time taken for the electrochemical signal to decay to 80% of its peak value (T_{80}). Electrochemical signal was produced by microinjection of 1mM of dopamine (DA) into the NTS. The decay time after drug or vehicle application of three microinjections of 5-HT was compared with microinjections three pre-drug or vehicle. Saline vehicle control; n=3, GBR 12909 1mg kg^{-1} n=3).

ns; non-significant; Compared to control with Students paired t-test.

4.6. Discussion

4.6.1. Main findings

The present study investigates the selectivity of a range of monoamine re-uptake inhibitors *in vivo*. The results can be used to determine the effective dose of these re-uptake inhibitors when they are to be used in experiments in order to identify an unknown electroactive species.

Citalopram, at a dose of 1 mg kg^{-1}, was found to cause selective inhibition of 5-HT uptake without affecting noradrenaline uptake in the NTS. Desipramine, at doses of 1mg kg^{-1}, was found to have the reverse selectivity, blocking noradrenaline but not 5-HT uptake in the NTS. Interestingly, reboxetine at a dose of 1mg kg^{-1} was found to inhibit both noradrenaline and 5-HT uptake, albeit to a lesser extent with respect to 5-HT. Decynium-22 (D-22; 600 μg kg-1) was also found to inhibit 5-HT reuptake, probably *via* blockade of PMAT not OCT3.

4.6.2. Drugs targeting 5-HT and noradrenaline transporters

Citalopram

Citalopram, at doses of 1 mg kg^{-1} (i.v.), inhibited the clearance of 5-HT in the NTS. The dose chosen did not affect the uptake of noradrenaline. This is consistent with literature in that citalopram has been shown to highly selective for 5-HT high--affinity, low-capacity transporter (5-HTT or SERT) compared to high-affinity, low-capacity noradrenaline transporter (NET). The original study Hyttel (1977) demonstrated that the uptake of noradrenaline into isolated mouse atria was affected only at micromolar concentrations compared to low nanomolar concentrations inhibiting 5-HT uptake into blood platelets. The same study also found that the metabolites of citalopram were also weak inhibitors of noradrenaline uptake but

potent 5-HT uptake inhibitors. Later, using rat lung slices, citalopram was also shown to be highly selectivity for the 5-HT transporter over noradrenaline transporter (Drew & Siddik, 1980). This study found that the concentrations up to 100μM of citalopram did not affect the uptake of noradrenaline, whereas 5-HT uptake was inhibited at 5nM. Studies using tissue from the CNS, in this case synaptosomes prepared from rat cortical tissue, found that citalopram had a selectivity ratio of approximately 4900 for 5-HT over noradrenaline (see Hyttel, 1982). Further evidence for high selectivity of citalopram for the 5-HT over the noradrenaline transporter was shown in brain slices, have a selectivty ratio of >1380 (Diggory *et al.*, 1980). These experiments using tissue *ex vivo* have been largely confirmed by transporter binding studies using expression systems *in vitro*. Using HEK293 (human embryonic kidney) cells expressing the 5-HT or the noradrenaline transporter similarly high ratio of 3500 was found (Tatsumi *et al.*, 1997). One early attempt at measuring noradrenaline uptake *in vivo* in the brains of rats and mice used 4,α-dimethyl-meta-tyramine to deplete noradrenaline, the effect of which can be blocked by re-uptake inhibitors (Carlsson *et al.*, 1969). Using the same method Hyttel (1977) found that noradrenaline depletion was unaffected by citalopram up to doses of 180 mg kg^{-1}, when administered subcutaneously (s.c.). The data from the present study confirms that citalopram is a potent an selective inhibitor of 5-HT re-uptake *in vivo*, which is in agreement with the literature, using both *in vitro, ex vivo* and indirect measurements of citalopram's ability to block re-uptake.

Fluoxetine

Interestingly, fluoxetine at a dose of 1 mg kg^{-1} (i.v.) failed to alter the removal of microinjected 5-HT from the NTS. This probably simply reflects the failure of this dose; 1 mg kg^{-1} may not allow a high enough concentration to reach in the brain to block the transporter. However, this is surprising as fluoxetine is known to have a lower K_i at the human 5-HT transporter than citalopram, 0.81 *vs.* 1.16nM Tatsumi *et al.* (1997). It would, therefore, be expected that i.v. doses equal to citalopram would be sufficient to inhibit the uptake of 5-HT. Indeed, there is evidence that fluoxetine blocks 5-HT re-uptake *in vivo* from a recent microdialysis study at doses of 1.5mg kg^{-1} when administered i.p. (Johansson *et al.*, 2012). The authors report an increase in

extracellular 5-HT concentration 30 min after administration of fluoxetine, which did not peak until 60 min post-administration. It could also be possible that the failure of 1mg kg^{-1} fluoxetine could be a result of differences in pharmacokinetics compared to citalopram. Fluoxetine could take longer to penetrate the CNS and given the short duration of the present experiment (approximately 15 min post administration), the full effects of the drug may not be seen.

Reboxetine

The present study showed that reboxetine 1 mg kg^{-1} (i.v.), as expected, blocked the high-affinity, low-capacity noradrenaline transporter, however at this dose reboxetine also inhibited the uptake of 5-HT. *In vitro*, reboxetine has a selectivity ratio of 81 for the noradrenaline transporter over the 5-HT transporter (Deecher *et al.*, 2006). This is poor when compared to citalopram and fluoxetine as they have selectively ratio of 3500 and 300 for the 5-HT transporter over the noradrenaline transporter (*in vitro*), respectively (Tatsumi *et al.*, 1997). *In vivo* one study using microdialysis to measure both dopamine and 5-HT efflux in the frontal cortex in response to tail pinch found that reboxetine significantly increased extracellular dopamine concentration, but had no effect on 5-HT (Page & Lucki, 2002). Conversely, an earlier study found that reboxetine caused a small increase in extracellular 5-HT in the striatum but dopamine was unaffected (Sacchetti *et al.*, 1999). From this data there seems to be regional differences in selectivity with reboxetine, however, in each case it could be possible that increased noradrenaline release feeds forward to increase dopamine and 5-HT release as microdialysis measures endogenous release. The present study confirms that reboxetine at doses of 1 mg kg^{-1} is not highly selective for noradrenaline uptake compared with 5-HT within the NTS.

Desipramine

Unlike reboxetine, desipramine 1 mg kg^{-1} (i.v.) did not significantly affect the uptake of 5-HT. Desipramine is known to have the higher affinity (*in vitro*) at the noradrenaline transporter (Tatsumi *et al.*, 1997) and less selectivity for noradrenaline

over 5-HT, having a selectivity ratio of 20 compared to reboxetine (80). However, *in vivo* doses of desipramine at 10 mg kg^{-1} acutely administered s.c. failed to have any effect on extracellular 5-HT concentration measured by microdialysis (Hajos-Korcsok *et al.*, 2000). This supports the possibility of higher selectivity in brain tissue than expression systems and agrees with the data from the present study that desipramine an extremely selective noradrenaline re-uptake inhibitor.

It should be noted that desipramine is also known to be a non-competitive inhibitor of OCT3 (Zhu *et al.*, 2010). However, data in the present study demonstrate that 5-HT is removed *via* PMAT, not OCT3 and this is supported by the desipramine having no effect on 5-HT clearance. It could, however, be possible that the reduction in noradrenaline removal rates seen with desipramine could be, in part, be due to blockade of OCT3.

GBR-12909

GBR-12909 failed to modify the clearance of exogenously applied dopamine in the NTS. Again this indicates that dose is not high enough. In this respect this study has shown that fluoxetine also at a dose of 1 mg kg^{-1} had no effect of the clearance of 5-HT. However, a dose of 0.75mg kg^{-1} i.v. has been previously shown to produce a potent and long lasting inhibition of the dopamine re-uptake in the striatum 3 seconds after administration (Espana *et al.*, 2008). Alternatively, the NTS may not express dopamine transporters in appreciable amounts necessary to remove the concentration of dopamine introduced exogenously in this experiment. Given that only limited roles for dopamine have been identified in the NTS (see INTRODUCTION 1.6.2) and given that 5-HT and noradrenaline are the predominate monoamine transmitters released within the NTS, extracellular dopamine concentrations are likely to be low. Expression of dopamine transporters may not be required at the same densities as 5-HT and noradrenaline transporters.

Table 4.1 Re-uptake inhibitor selectivity: Summary of results

Compound	Substrate		Selective at Dose (1mg kg^{-1})
	5-HT	NA	
Citalopram	✔	✖	✔
Desipramine	✖	✔	✔
Reboxetine	✔	✔	✖
Fluoxetine	✖	–	–

4.6.3. Drugs targeting OCT3 and PMAT

Decynium-22

Decynium-22 (D-22; 600µg kg^{-1}; i.v) significantly reduced the clearance of exogenously applied 5-HT in the NTS. D-22 is a potent inhibitor of OCT3 and PMAT (Schomig *et al.*, 1993, Duan & Wang, 2010). It has also been shown that D-22 is readily centrally penetrant (Horton *et al.*, 2013). The present data indicates that OCT3 and/or PMAT are responsible for the removal of 5-HT from the extracellular space within the NTS. D-22 has previously been shown to block uptake of exogenous 5-HT *in vivo*, in the hippocampus, only when 5-HT transporter function is comprised by genetic deletion (Baganz *et al.*, 2008). This may represent regional differences in how extracellular 5-HT is metabolised. Hippocampal uptake of 5-HT may rely on more heavily on the 5-HT transporter and OCT3/PMAT takes over at higher concentrations of 5-HT. The data indicates that the uptake mechanisms are more evenly weighted within the NTS

Corticosterone

Corticosterone failed to affect the uptake of 5-HT when administered i.v. at doses from 1-10mg kg^{-1}. Corticosterone is an inhibitor of OCT3 with an IC_{50} 0.29μM when measured in vitro in an HEK cell expression system (Hayer-Zillgen et al., 2002). Corticosterone is a selective inhibitor for OCT3, whereas PMAT is insensitive to steroids (Gasser et al., 2006, Duan & Wang, 2010, Hill & Gasser, 2013). This data would suggest that the inhibition of 5-HT uptake by D-22 is a result of blockade PMAT not OCT3.

4.6.4. Conclusion

The present experiments were to determine an effective dose for a range of re-uptake inhibitors in vivo. It was found that citalopram and desipramine are the drugs of choice to selectively manipulate the 5-HT and noradrenaline transporter because they were selective at doses of 1mg kg^{-1} within the NTS (see table 4.1). Further, PMAT was also shown to be responsible for the removal of 5-HT from the extracellular space, even with 5-HT transporter function intact.

4.6.5. Future experiments

Determine the effect of D-22 on the re-uptake of noradrenaline. PMAT is capable of removing noradrenaline from the extracellular medium, however PMAT is 70% more effective at removing 5-HT over noradrenaline when measured *in* vitro (Duan & Wang, 2010). Doses effective at inhibiting 5-HT removal may fail to modify noradrenaline uptake. With carful dosing it may be possible to use D-22 to selectively inhibit 5-HT uptake *via* PMAT while noradrenaline remains unchanged.

5. ACTIVATION OF CARDIOVASCULAR AFFERENTS CAUSE 5-HT RELEASE IN THE NTS DETECTED DIRECTLY BY FAST-CYCLIC VOLTAMMETRY

5.1. Introduction

Although glutamate is the principal neurotransmitter in the NTS there are many other transmitters involved in modulating cardiovascular reflexes (see INTRODUCTION 1.6.2). One of these proposed modulating transmitters is 5-HT, which has been identified to play an important role in many facets of cardiovascular regulation (see Ramage & Villalon, 2008).The extensive innervation of the NTS by 5-HT afferents is consistent with such a role. First, there is evidence of 5-HT terminals originating centrally from raphé magnus and dorsal raphé (Schaffar *et al.*, 1988, Sim & Joseph, 1992). Second, 5-HT-containing terminals from vagal afferents (Sykes *et al.*, 1994) originating from the nodose ganglion (Gaudin-Chazal *et al.*, 1982, Nosjean *et al.*, 1990) as well as the petrosal ganglion (Thor *et al.*, 1988) are found in the NTS. Third, there is evidence of 5-HT-containing interneurons within the NTS (Calza *et al.*, 1985). However, it should be noted that there are no further corroborating reports of 5-HT interneurons in NTS.

There is also extensive expression of 5-HT receptor subtypes within the NTS itself (see INTRODUCTION 1.3). Of these expressed subtypes, 5-HT_3 (Jeggo *et al.*, 2005) and 5-HT_7 (Oskutyte *et al.*, 2009)receptors have been shown to play a physiological role in cardiovascular reflex pathways, at least *in vivo*. Both studies showed that antagonists of 5-HT receptors reduce the response in NTS cells when activated by vagal stimulation. This strongly suggests that 5-HT is released from vagal afferents.

Until now there have been no attempts to measure this release of 5-HT directly. This is partly due to the technological limitations of microdialysis, where large probe surface areas preclude sampling from a discrete nucleus, even in larger animals. Indeed, where microdialysis has been attempted in the NTS of the cat or piglet, dialysate recovered was not often confined to the nucleus (Allchin *et al.*, 1994,

Waters *et al.*, 1997, Potts & Fuchs, 2001). However, fast-cyclic voltammetry is widely used to investigate dopamine release and re-uptake dynamics *in vivo* with an extremely small electrode allowing sampling from small brain nuclei, such as the NTS (see INTRODUCTION 1.7). The present study will use the technological advances afforded by voltammetry to detect any electrochemical changes within the NTS as a result of vagal stimulation (figure 5.1). Additionally, the advantage of performing such experiments within a whole animal allows monitoring of end organ responses simultaneously with neurotransmitter dynamics in the region of interest. With this unique experimental approach this study aims to correlate neurochemical changes with cardiovascular outcomes.

5.2. Specific Aims

- Assess the suitability of voltammetry to detect 5-HT release in a small brain nucleus such as the NTS. Determine the selectivity profile of the trapezoidal ramp and differential scans (see METHODS 2.1.5) for 5-HT and other interfering electroactive molecules commonly found in the brain.

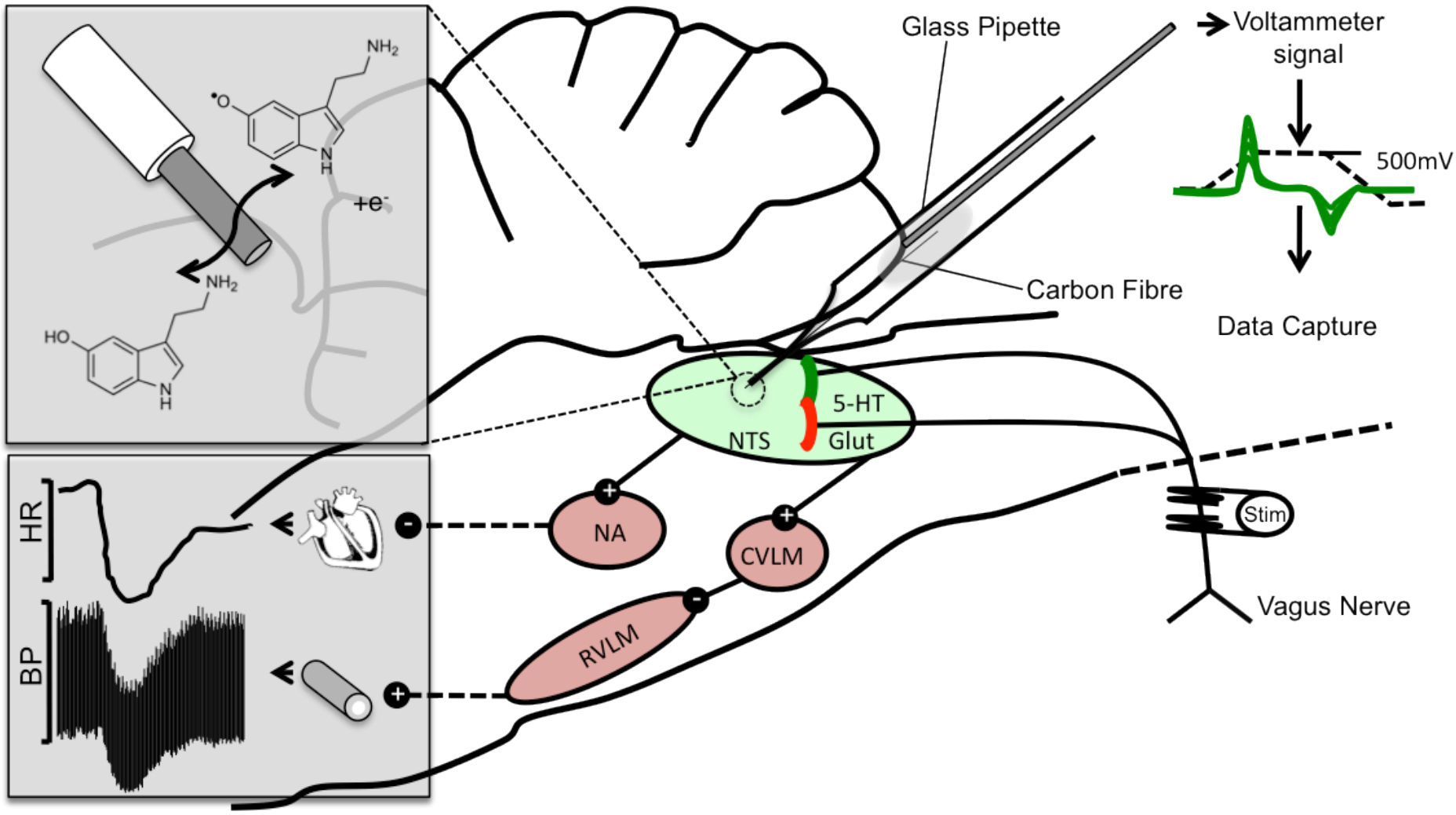
- Determine if an electrochemical signal is produced in the NTS as a result of vagal stimulation and identify the species responsible for this signal using the four criteria set down for the purpose by (Phillips & Wightman, 2003)

- Determine the source of the electrochemical species. Is the release from terminals arising from central locations or peripherally from vagal afferents?

- Investigate whether activation of cardiovascular reflexes causes release of electroactive substances within the NTS.

Figure 5.1 Diagrammatic representation of the *in vivo* electrochemistry preparation

Schematic representation of a sagittal section of the brainstem showing the nucleus tractus solitarius (NTS), nucleus ambiguus (NA), caudal ventrolateral medulla (CVLM) and the rostral ventrolateral medulla (RVLM). Vagal afferent fibre terminals containing glutamate and 5-HT are shown synapsing in the NTS together with the carbon fibre microelectrode with the steps for voltammetric recording. The top left inset shows the molecular scheme of the oxidation and re-reduction reaction occurring on the surface of the carbon fibre used for 5-HT detection. The bottom left insert shows the effects of vagal afferent stimulation on heart rate and blood pressure. Activation of ambigual neurons increases parasympathetic drive (-) to the heart, causing a bradycardia. Activation of the CVLM inhibits the RVLM, which then reduces sympathetic drive (+) to the arterial blood vessels. This sympathetic withdrawal causes vasodilation and thus a fall in blood pressure.


Glass Pipette
Voltammeter signal
500mV
Data Capture
Carbon Fibre
NH2
HO
+e-
5-HT
Glut
NTS
NA
CVLM
RVLM
Stim
Vagus Nerve
HR
BP

5.3. Results: Selectivity

Figure 5.2 shows voltammograms recorded *in vitro* in the presence of electroactive substances commonly found in the brain. Selectivity between the analyte of interest (5-HT) and possible interfering substances was determined *in vitro* and a 'rejection ratio' was determined to compare selectivity. Rejection ratio refers to the difference between the current produced at the same concentration between two electroactive compounds. For example if a rejection ratio was 50 to 1 between compound A and B then it would require 50 times the concentration of compound A to produce the same current change as compound B.

5-HT at a concentration of 0.1μM produces a total increase in faradic current of 70nA. When compared with other electroactive neurotransmitters tested this gives a rejection ratio of approximately 8 to 1 for noradrenaline (9nA at 0.1μM) and approximately 100 to 1 for dopamine (7nA at 1μM). Ascorbic acid, commonly found packaged in 5-HT containing vesicles, was found to produce no current changes when sampled at the points of maximum current change for 5-HT, even at concentrations 1000 times higher. 5-HIAA, which is a direct metabolite of 5-HT, was found to produce currents of 22nA at concentrations of 10μM. This gave a rejection ratio of approximately 320:1 over 5-HT.

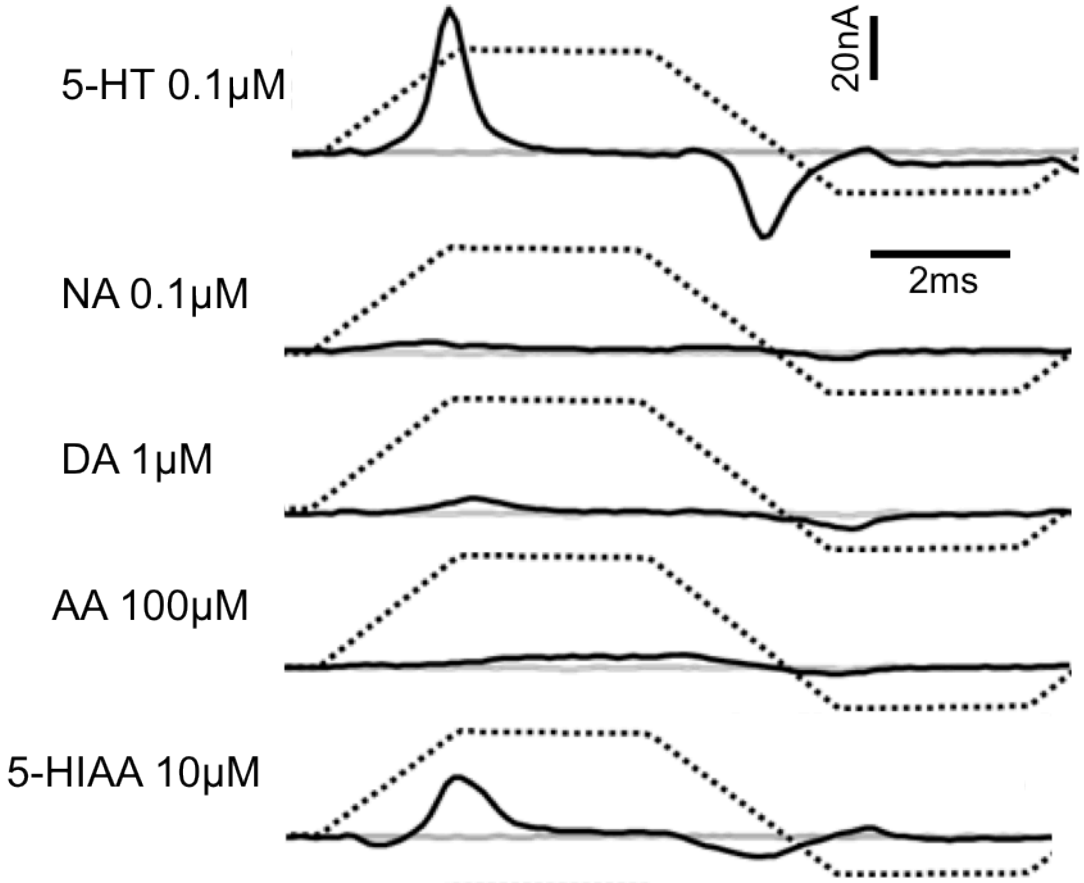


Figure 5.2 Selectivity over other electroactive substances

Voltammograms recorded 0.1mM phosphate buffered saline with addition of the following electroactive substances known to found in in the brain. Active scan voltage ramp is shown by dashed line. 5-HT, 5-HIAA; 5-Hydroxyindoleacetic acid, NA; Noradrenaline, DA; Dopamine, AA; Ascorbic acid.

5.4. Results: Electrochemistry

As shown in Figure 5.3 the faradic current profile obtained *in vivo* in the NTS (figure 5.4) immediately after electrical stimulation of the vagus nerve (20Hz, 10x threshold) broadly overlies the faradic current profile recorded *in vitro* (0.01M phosphate buffered saline) in the presence of 30nM 5-HT. The peak of the oxidation current both *in vivo* and *in vitro* occurs at voltages of 500mV. The re-reduction current starts to occur at the same position of the negative voltage ramp and covers the same range of voltages, however the peak is slightly delayed *in vivo*. Additionally, the oxidation peak is typically larger than the re-reduction peak *in vivo* and this is reversed *in vitro*.

It is expected that there will be slight differences between the faradic current profile recorded *in vitro* and that recorded *in vitro*. Firstly, electrical impedance of the recording medium will be different; brain tissue will have higher impedance than saline, which will result in a lower effective voltage being delivered to the tip of the electrode. This difference will cause a rightward shift in the oxidation and re-reduction peak as lower voltages will be applied at the same time point of the scan, thus delaying the oxidation and re-reduction of 5-HT. Additionally, there are several electroactive species present in the extracellular fluid *in vivo* that could interact with 5-HT directly to change its oxidation state after it is released form the nerve terminal. The increased re-reduction peak could be the result of 5-HT oxidised by means other than the voltammetric scan. The already oxidised 5-HT would then diffuse to the electrode and be re-reduced as the electrode cannot distinguish between 5-HT oxidised by the voltammeter and by other means.

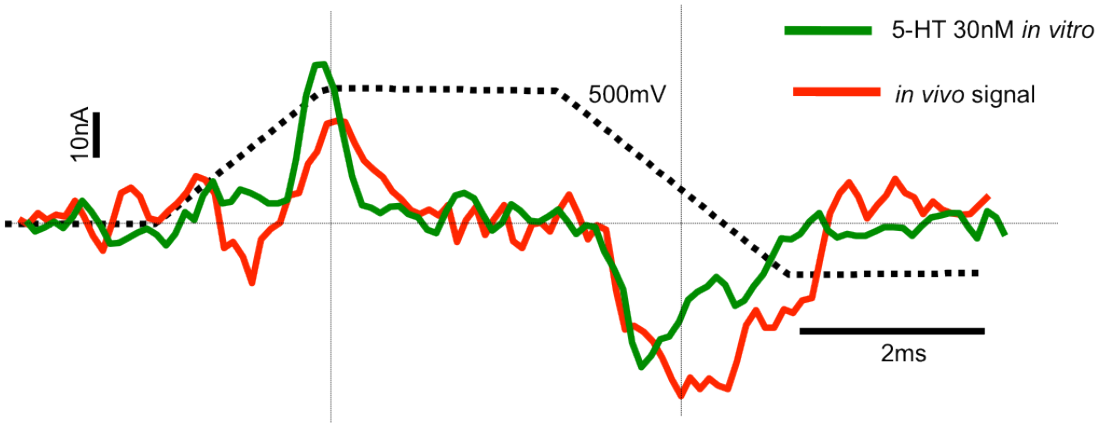


Figure 5.3 Comparisons of voltammograms

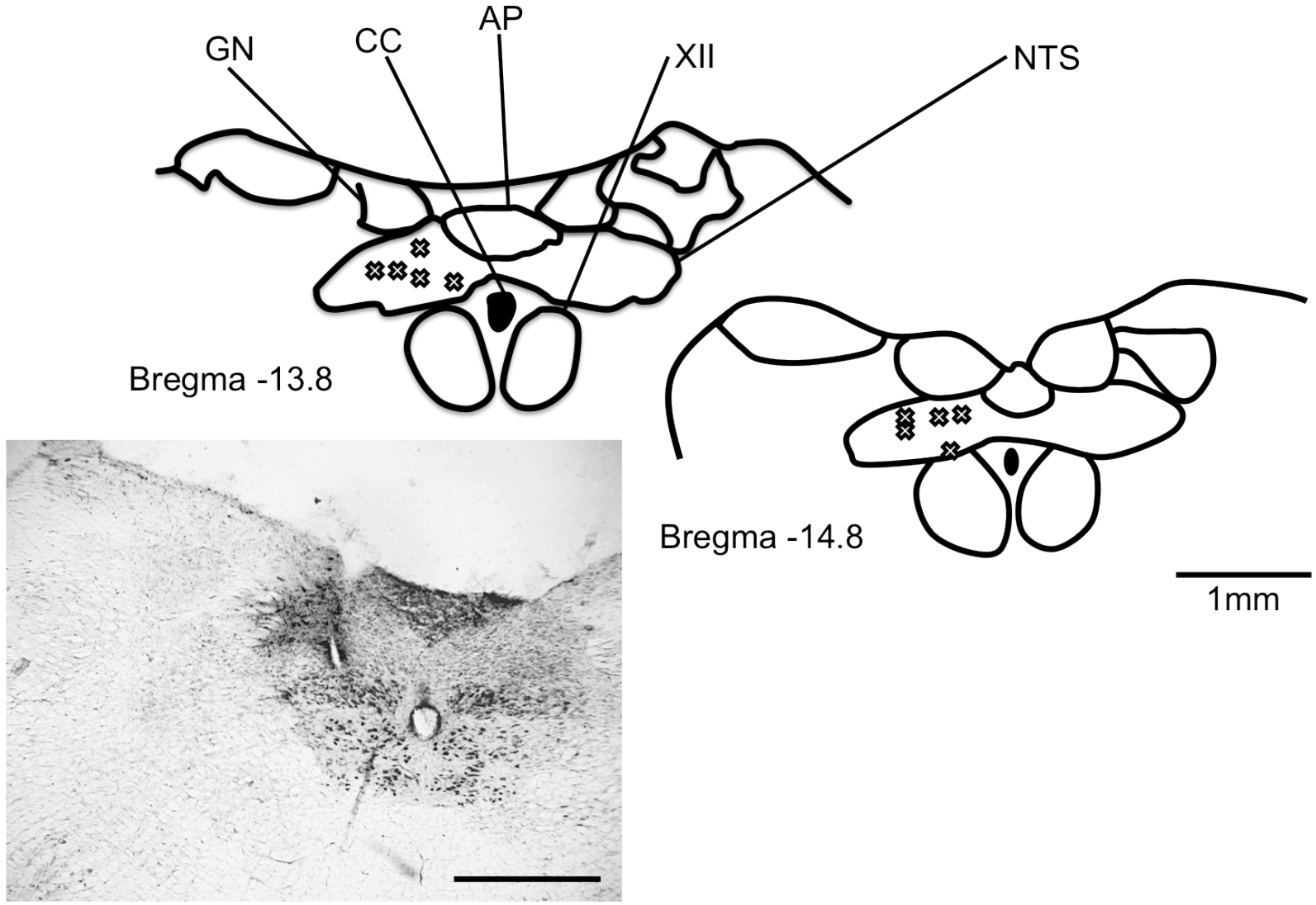
Typical faradic current traces (voltammograms) recorded *in vivo* after electrical stimulation of the vagus overlaid with a current trace recorded *in vitro* in the presence of 30 nM 5-HT solution in phosphate buffered saline. The dashed line indicates the voltage ramp applied to the carbon fibre microelectrode. The vertical lines indicate the maximum of the oxidation and re-reduction currents recorded in vivo. The total current passed (oxidation + re-reduction) is used to calculate the extracellular concentration change.

Figure 5.4 Histology

Recording sites with the NTS are shown for a sample of 10 experiments (✖ denotes recording site). Electrode tip locations were determined by current-induced lesions were recovered histologically after the experiment that were subsequently mapped to coordinates using a rat brain atlas.

Inset shows an example of a typical photomicrograph of a coronal section of the rat brainstem showing the electrode lesion in the NTS. The tissue was stained with neutral red and photographed in black and white for increased contrast. Scale bar is 1mm

AP; Area postrema
CC; Central canal
GN; gracile nucleus
NTS; nucleus of the solitary tract
XII; hypoglossal motor nucleus


GN
CC
AP
XII
NTS
Bregma -13.8
Bregma -14.8
1mm

5.5. Results: Vagal stimulation frequency response

Stimulus intensity for electrical stimulation of the vagus nerve was calculated as multiples of the threshold at which evoked neuronal spike activity is detected in the NTS. When stimulated at threshold (1x threshold) frequencies of 5- 50Hz produced changes in extracellular 5-HT of 4.6 ± 1.5, 4.4 ± 1.1, 5.8 ± 2.3 and 6.8 ± 3.0nM, respectively (figure 5.6). All values expressed as multiples of threshold. This was not significantly different from the baseline variability, which was calculated to be 0.3 ± 2.0 nM over the same time period as the peak change in 5-HT concentration following stimulation.

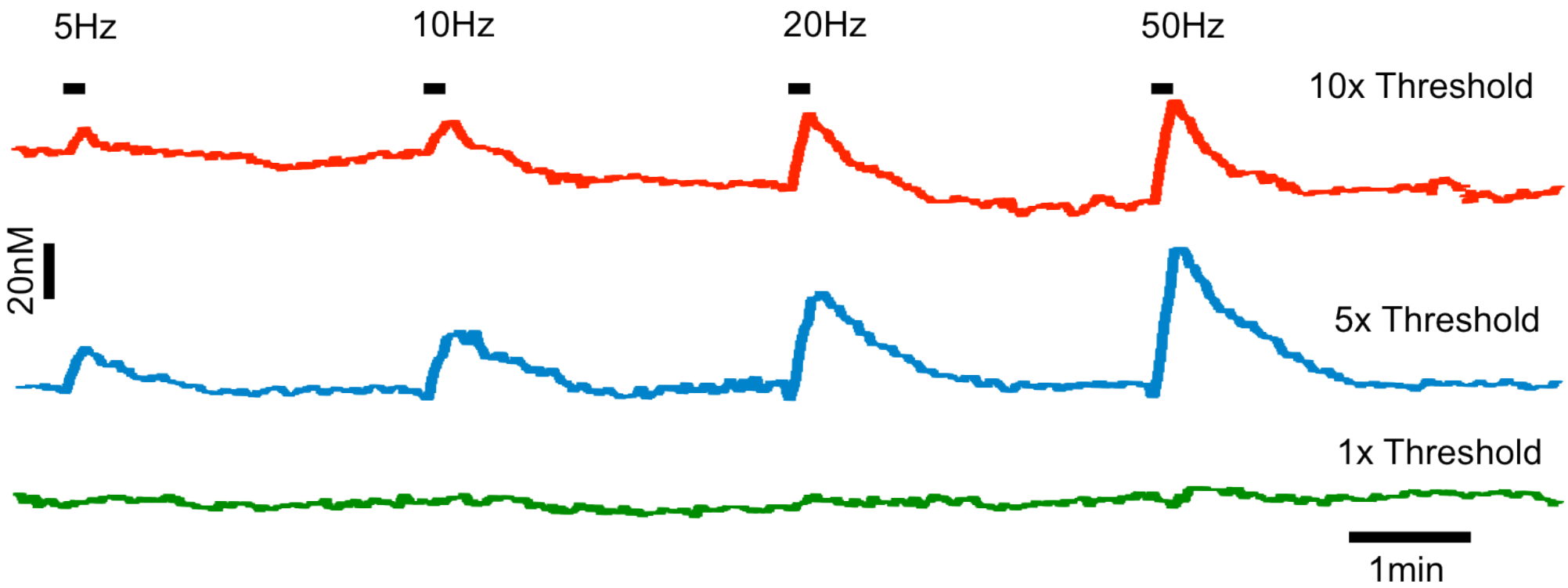
When stimulated at threshold (5x threshold) frequencies of 5- 50Hz produced changes in extracellular 5-HT of 12.0 ± 4.6, 24.5 ± 6.4, 32.0 ± 11.0 and 44.1 ± 15.6nM, respectively (Figure 5.6). Frequencies of 20 and 50Hz produce changes in extracellular 5-HT concentration that were significantly different from baseline (P<0.05; 1-Way ANOVA; compared with Fisher's LSD test).

When stimulated at threshold (10x threshold) frequencies of 5- 50Hz produced changes in extracellular 5-HT of 12.1 ± 5.2, 17.9 ± 7.2, 30.0 ± 7.8 and 50.3 ± 10.8 nM, respectively (Figure 5.6). Again, frequencies of 20 and 50Hz produce changes in extracellular 5-HT concentration that were significantly different from baseline (P<0.01; 1-Way ANOVA; compared with Fisher's LSD test).

Representative experimental traces from one subject contributing to the data described above are shown in figure 5.5.

Figure 5.5 Vagal stimulation frequency response: Experimental Traces

Anaesthetised artificially ventilated, neuromuscular-blocked male rat: traces showing the change in extracellular 5-HT in response to electrical stimulation of the central end of the left vagus (1ms pulse-width, 10s duration) at 3 min intervals at different frequencies and thresholds. Threshold range was between 70-110 μA. Threshold was defined as the current at which evoked neuronal activity was detected in the NTS.


5Hz
10Hz
20Hz
50Hz
10x Threshold
20nM
5x Threshold
1x Threshold
1min

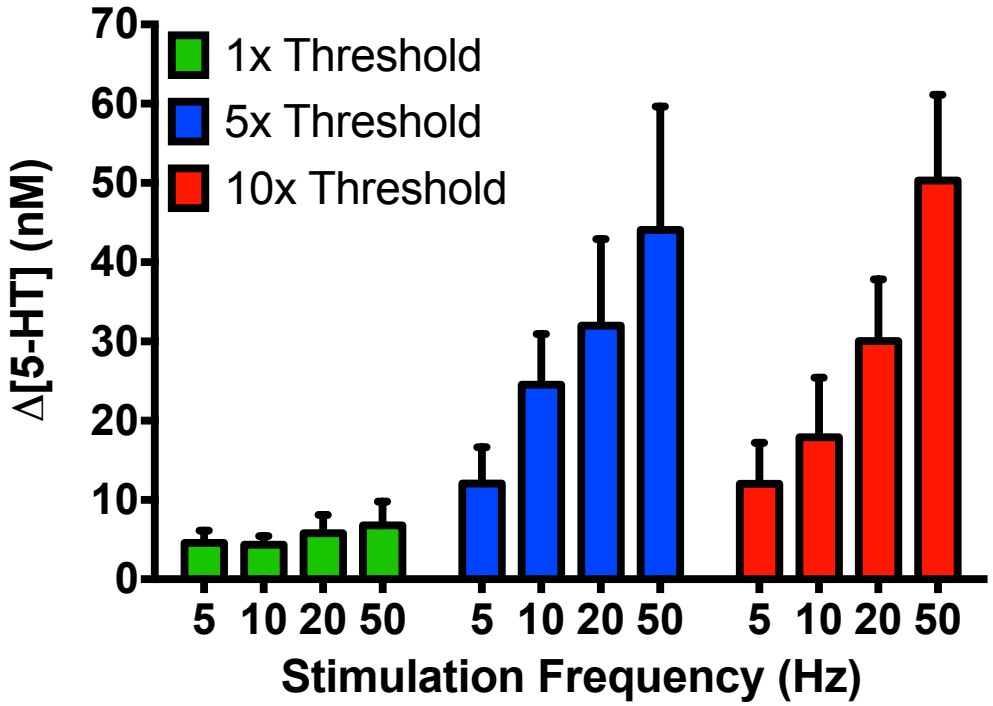


Figure 5.6 Frequency response: Histogram

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the different frequencies and thresholds for electrical stimulation of the central end of the left vagus (1ms pulse-width, 10s duration) at 3 min intervals at different frequencies and thresholds (threshold range was between 70-110 μA) on changes (Δ) in the 5-HT concentration in the NTS as detected by fast cyclic voltammetry. Each column is the mean data from 5 rats. Stimulation levels (x threshold) were randomised from rat to rat. The error bars show the s.e.m.

5.6. Results: Effect of kynurenate and cadmium

Haemodynamic baseline data

Baseline heart rate and blood pressure was measure 30s prior to commencement of the experimental protocol. The 10mM kynurenate experimental group had a baseline blood pressure of 98 ± 5 mmHg and a baseline heart rate of 387 ± 12 bpm. The 50mM kynurenate experimental group had a baseline blood pressure of 102 ± 8 mmHg and a baseline heart rate of 397 ± 12 bpm. The 50mM cadmium chloride experimental group had a baseline blood pressure of 101 ± 8 mmHg and a baseline heart rate of 401 ± 16 bpm. The vehicle control (30µl, 0.01M PBS) experimental group had a baseline blood pressure of 103 ± 5 mmHg and a baseline heart rate of 395 ± 16 bpm.

5-HT release baseline data

Three stimulations were performed prior to the application of the test drug or vehicle in each experimental group. The average absolute increases of extracellular 5-HT measured in each of these groups were as follows: 10mM KYN; 33 ± 9nM, 50mM KYN; 28 ± 4nM, cadmium; 29 ±5nM and vehicle; 22 ± 7nM.

Effect of kynurenate

Topical application of 10mM kynurenate (KYN) produced an increase in baseline of 10 ± 2 bpm and 12 ± 1 mmHg in heart rate and blood pressure, respectively. The higher dose, 50mM, caused an increase in baseline of 26 ± 8 bpm and 29 ± 2 mmHg in heart rate and blood pressure, respectively. Topical application of vehicle caused a change of -5 ± 6 bpm and 2 ± 4 mmHg in heart rate and blood pressure, respectively.

In the presence of kynurenate the extracellular increase in 5-HT produced by vagal stimulation (20Hz, 10x threshold) was attenuated (figure 5.9). At doses of 10mM the pre-drug change in extracellular 5-HT was reduced by 51.8 ±5.0 %. Equally, at the

higher dose of 50mM the change in extracellular 5-HT was reduced 51.3 ±5.3 %. Both were changes significantly different from those in subjects receiving time-matched vehicle (PBS; 0.01M, topical), which only caused a change of -0.44 ± 2.8% from pre-drug control stimulations.

In the presence of 10mM kynurenate vagal stimulation produced a decrease in mean blood pressure of 18.6 ± 1.5 mmHg. This is not significantly different from vehicle control, in the presence of which vagal stimulation produced a decrease of -28.8 ± 5.7 mmHg. However, with the higher dose of 50mM, vagal stimulation now caused a rise in blood pressure of 12.4 ± 1.2 mmHg. This was significantly different from vehicle control.

In the presence of 10mM kynurenate vagal stimulation produced a decrease in heart rate of -22.7 ± 8.9 bpm. This is not significantly different from vehicle control, which produced a decrease of -28.8 ± 5.7 mmHg. However, with the higher dose of 50mM vagal stimulation now caused a rise in heart rate of 6.2 ± 3.8 bpm. This was significantly different from vehicle control.

Figure 5.7 shows representative experimental traces of the effect of kynurenate on both baseline heart rate and blood pressure as well as changes to extracellular 5-HT, heart rate and blood pressure during vagal stimulation.

Effect of cadmium chloride

Topical application of 50mM cadmium chloride ($CdCl_2$) produced an increase in baseline heart rate and blood pressure of 37 ± 11 bpm and 41 ± 13 mmHg, respectively.

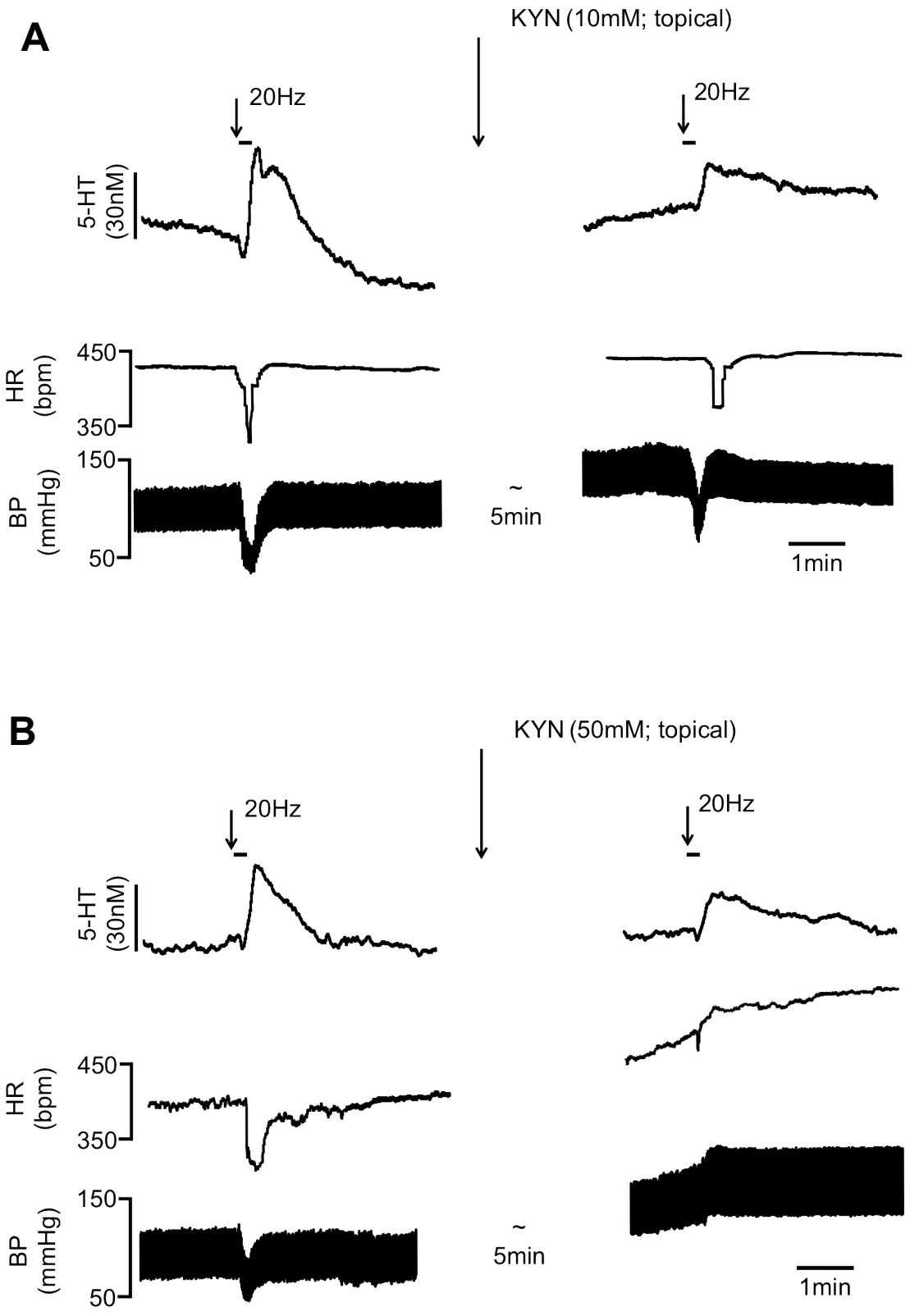
In the presence of 50mM cadmium chloride the extracellular increase in 5-HT caused by vagal simulation (20Hz, 10x threshold) was reduced by 92.0 ± 14.2% when compared to pre-drug controls (figure 5.9). This was significantly different from time-matched vehicle control (PBS; 0.01M, topical), which only caused a change of -0.44 ± 2.8% when compared to pre-drug control stimulations.

Topical application of cadmium chloride also attenuated both the reduction in mean blood pressure and heart rate caused by vagal simulation. In the presence of cadmium chloride blood pressure was now only reduced by 7.6 ± 1.5 mmHg and heat rate only reduced by -7.2 ± 3.4 bpm. Again, both these changes were significantly different when compared to the change recorded in the presence of vehicle control (heart rate; -35.2 ± 3.4 bpm and blood pressure -28.8 ± 5.7 mmHg).

Figure 5.8 shows representative experimental traces of the effect of cadmium chloride on both baseline heart rate and blood pressure as well as changes to extracellular 5-HT, heart rate and blood pressure during vagal stimulation.

Figure 5.7 Effect of kynurenate: Experimental Traces

Two anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of topical (30µl) application of kynurenate (a) 10mM and (b) 50mM on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 10s at 10X threshold) of the central end of the left vagus.


A
KYN (10mM; topical)
20Hz
20Hz
5-HT (30nM)
HR (bpm)
450
350
BP (mmHg)
150
50
~ 5min
1min
B
KYN (50mM; topical)
20Hz
20Hz
5-HT (30nM)
HR (bpm)
450
350
BP (mmHg)
150
50
~ 5min
1min

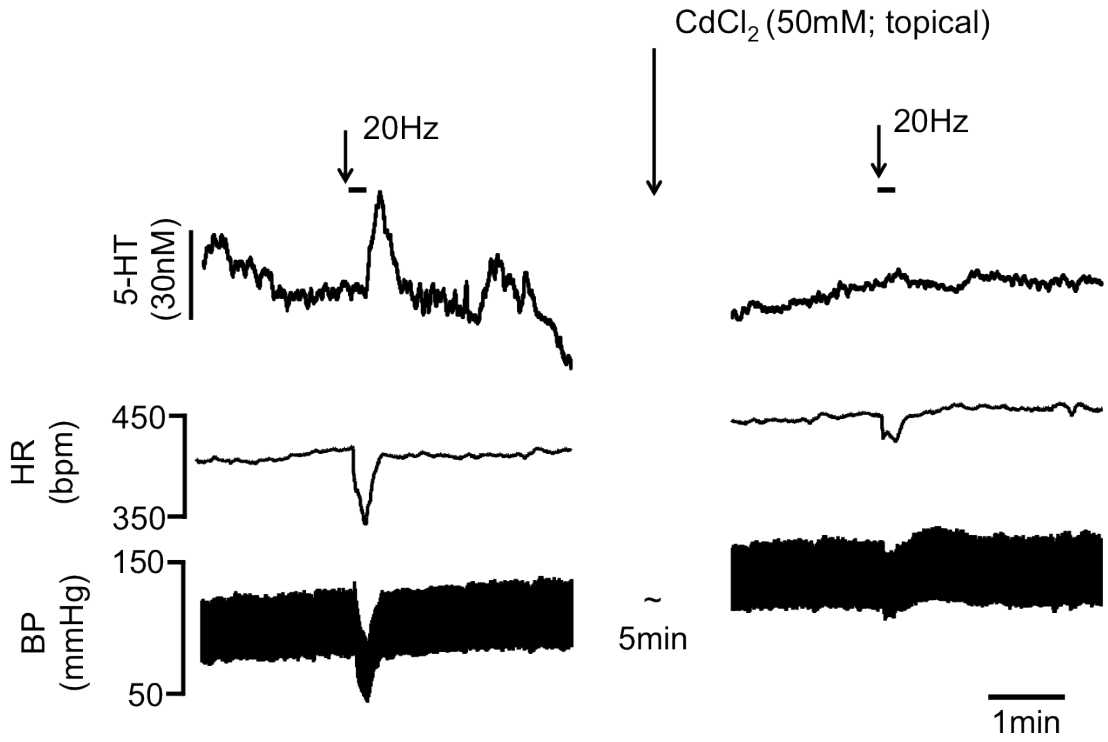
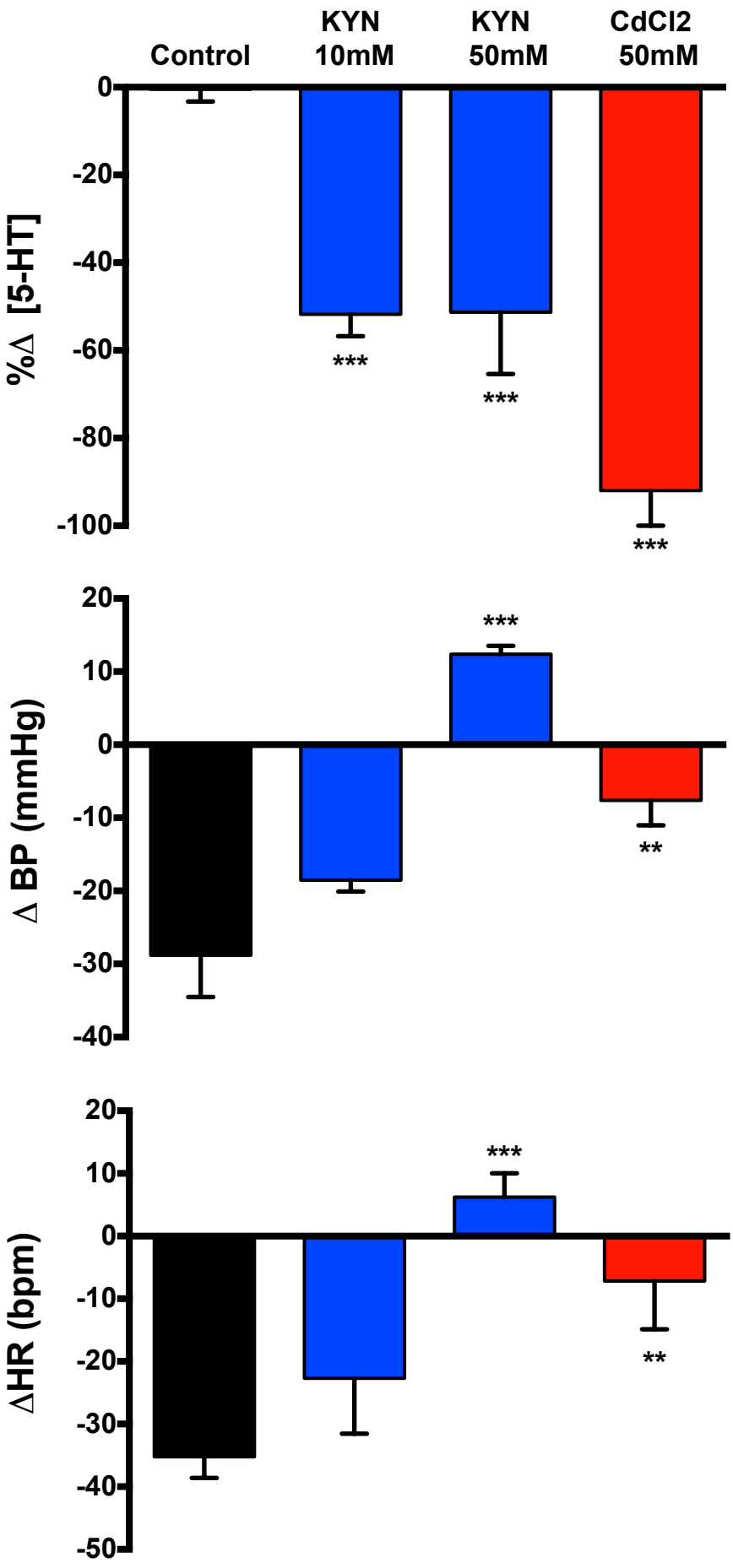


Figure 5.8 Effect of cadmium: Experimental Traces

An anaesthetised artificially ventilated, neuromuscular -blocked male rat showing the effect of topical (30µl) application of 50mM cadmium chloride on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 10s at 10X threshold) of the central end of the left vagus.

Figure 5.9 Effect of kynurenate and $CdCl_2$: Combined data

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of topically (30 µl) applied kynurenate (KYN; 10 & 50 mM, n=6 & 7) and cadmium chloride (CdCl2; 50 mM, n=5) or 0.01M phosphate buffered saline (control; n=7) on increases in the 5-HT concentration in the NTS and the decreases in mean arterial blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 10s at 10X threshold) of the central end of the left vagus. The bars show the s.e.m. These changes were compared to control using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. ** P<0.01; *** P<0.01


Control
KYN 10mM
KYN 50mM
CdCl2 50mM
%Δ [5-HT]
0
-20
-40
-60
-80
-100
***
***
***
Δ BP (mmHg)
20
10
0
-10
-20
-30
-40
***
**
ΔHR (bpm)
20
10
0
-10
-20
-30
-40
-50
***
**

5.7. Results: effect of metabolic inhibitors PCPA and AMPT

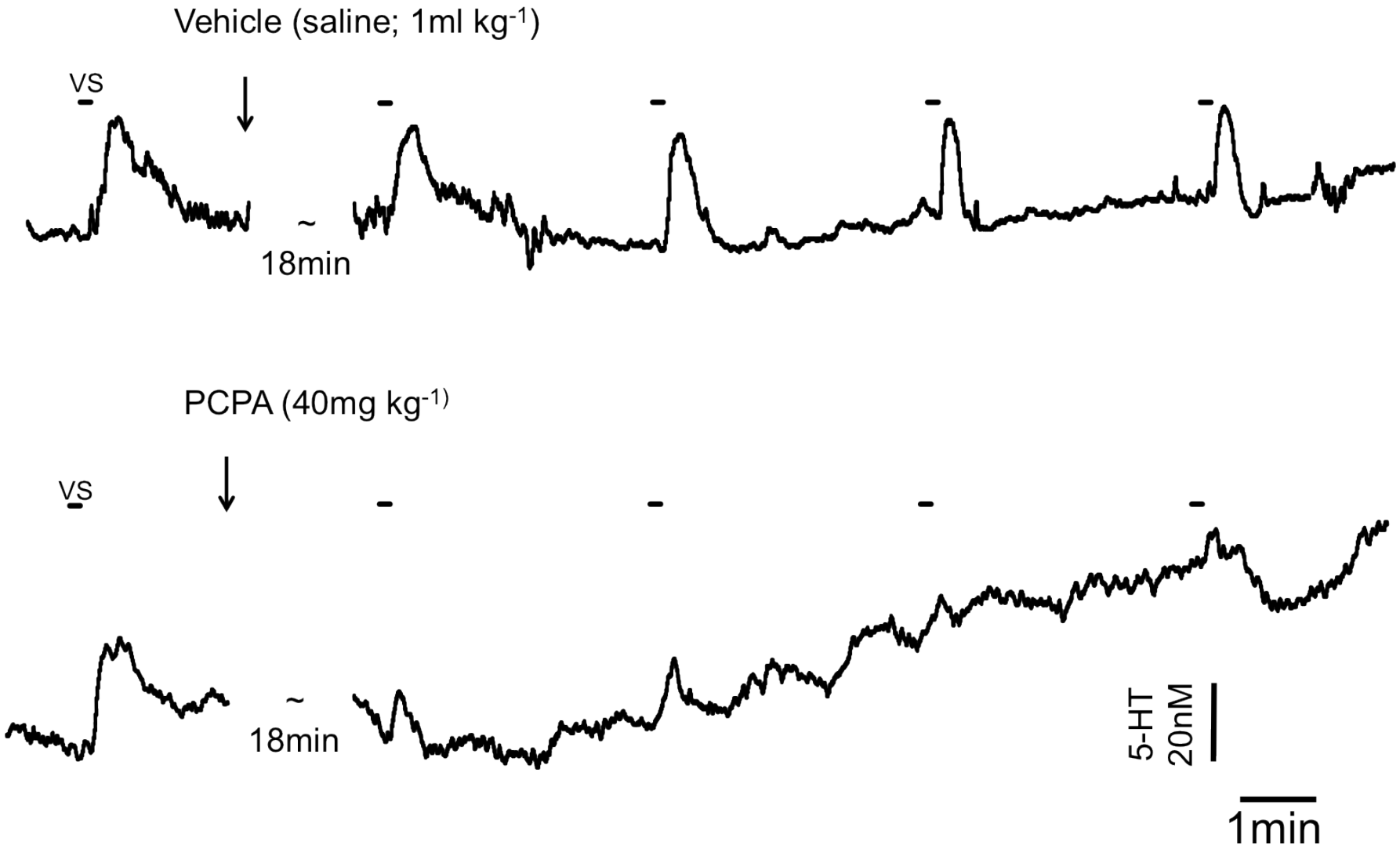
Intravenous application of the tryptophan hydroxylase inhibitor PCPA (40mg kg^{-1}; n= 4) caused a significant (P<0.05) reduction in the expected increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) over time (figure 5.10). The increase in extracellular 5-HT caused by first the simulation after application of PCPA was reduced to 60 ± 4 % of the pre-drug control value. This was significantly different from the change at the same time point after administration of saline control (1ml kg^{-1}; n=6), which was 100 ± 8 % of the pre-drug control value (figure 5.11). The significant PCPA-induced reduction persisted from this time point and reached a maximum difference at 30min (41 ± 6 % of the pre-dug control). However, by 33 min the significant reduction had recovered to 66 ± 13% of the pre-drug control, which was not significant from saline control at the same time point (90.3 ± 9%). By the end of the experimental protocol, 45min after drug administration, the change in extracellular 5-HT concentration had recovered to 74 ± 9% of pre-drug control values.

Intravenous application of the tyrosine hydroxylase inhibitor AMPT (200mg kg^{-1}; n= 4) caused no significant change in the expected increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) over time. The increase extracellular 5-HT caused did not differ significantly from control at any time point during the experimental protocol.

There was no significant differences in hypotension or bradycardia produced by vagal stimulation between the AMPT, PCPA or vehicle control groups at any time point during the experimental protocol. There was, however, a generalised increase in the bradycardia over all groups as time progressed. By the final time point (45min) the heart rate changes had increased to 132 ± 18 in control, 137 ± 13 in the PCPA group and 187 ± 43% of pre-drug/vehicle values. Percentage changes were used here due to high intraexperimental variability during the extended recording times.

Figure 5.10 Effect of PCPA: Experimental Traces

An anaesthetised artificially ventilated, neuromuscular -blocked male rats showing the effect PCPA (40 mg kg-1; i.v., bottom trace) compared with vehicle control (saline; top trace) on the increases in the 5-HT concentration in the NTS evoked by a series of electrical stimulations (20Hz, 1ms for 10s at 10X threshold) of the central end of the left vagus given at 3 min intervals as shown by the line (VS) about the traces. At the beginning of both sets of traces the control evoked increase in 5-HT is shown for that rat followed a by a gap of 18 min.


Vehicle (saline; 1ml kg-1)
VS
18min
PCPA (40mg kg-1)
VS
18min
5-HT
20nM
1min

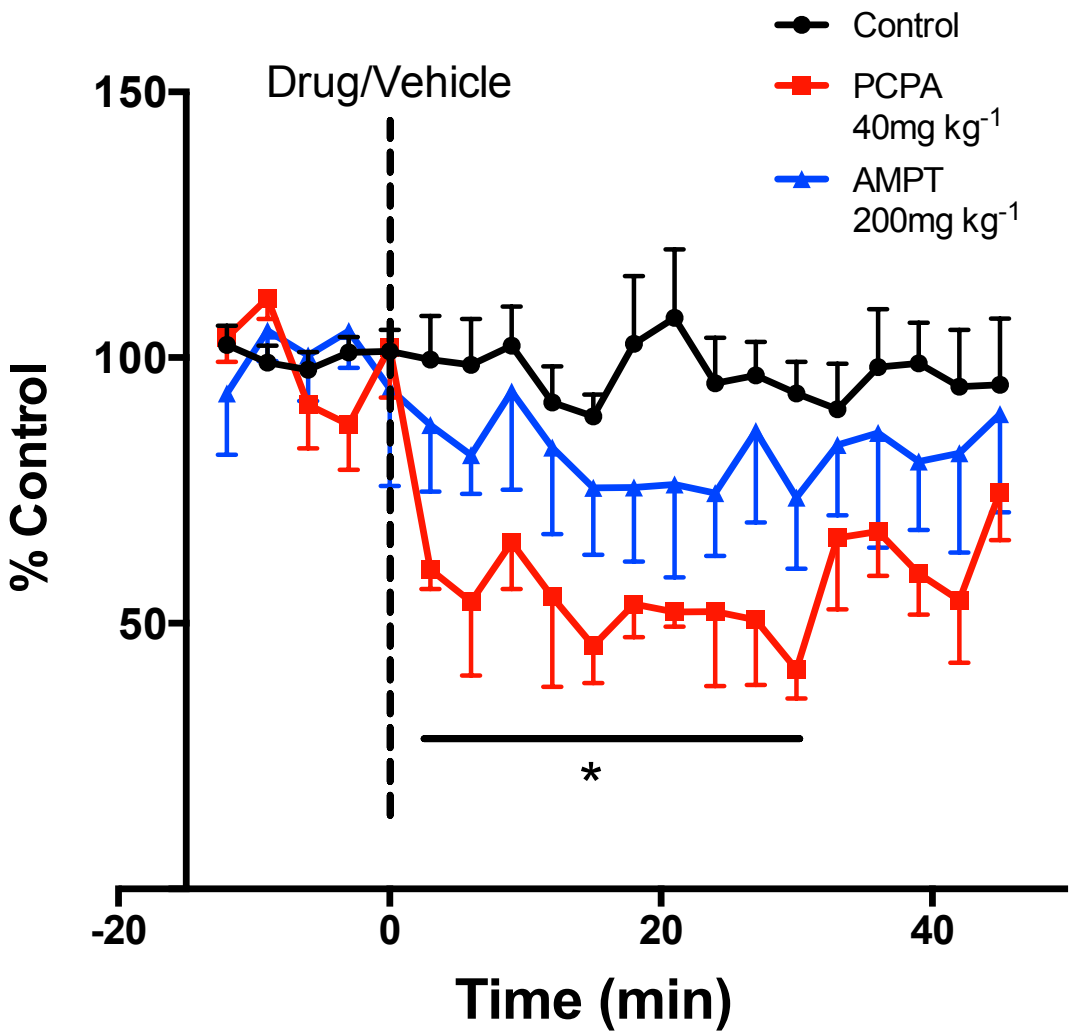


Figure 5.11 Effect of PCPA and AMPT: Graph

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: Graph comparing the effects of i.v. saline (control; 1ml kg^{-1}, n=6), PCPA (40mg kg^{-1}, i.v, n=4) and AMPT (200mg kg^{-1}, i.v., n=4) on increases in the 5-HT concentration in the NTS evoked by electrical stimulation (20Hz, 1ms, 10s at 10X threshold) of the central end of the left vagus. The bars show the s.e.m. These changes were compared to control using a 2-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. * $P<0.05$;

5.8. Results: Effect of drugs inhibiting re-uptake systems

Baseline data

Baseline heart rate and blood pressure were measured 30s prior to commencement of the experimental protocol. Additionally, three stimulations were performed prior to the application of the test drug or vehicle in each experimental group. The average absolute increases of extracellular 5-HT measured in each of these groups. See table 5.1 for a comprehensive list.

Effect of re-uptake inhibitors on baseline haemodynamic values

Injection of all re-uptake inhibitors was associated with a small increase in baseline blood pressure and heart rate, with the exception of D-22. Table 5.1 contains the complete data. Injection of D-22 (600μg kg^{-1}, i.v) caused a decrease in blood pressure of -49 ± 7mmHg and an increase in heart rate of 86 ± 15 bpm. In a separate set of experiments (figure 5.16) the decrease in blood pressure was mimicked by injection of the ganglion blockade by chlorisondamine (1mg kg^{-1}, n=3). This caused a decrease in blood pressure of 44 ± 8mmHg, which was not significantly different from that caused by D-22. In these experiments, evoked 5-HT changed by 5.2 ± 10%, which was significantly different from the DMSO control in this experiment (-3.1 ± 2.6%).

Effect of re-uptake inhibitors on vagal stimulation

Intravenous application of the selective 5-HT transporter inhibitor citalopram (1mg kg^{-1}; n= 6, figure 5.12) caused no significant change in the evoked increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) when compared to application of vehicle (DMSO, 1ml kg^{-1}; n=6) control, which caused a change of -3.1 ± 2.6%. After administration of citalopram the change over pre-drug control stimulations was only -1.3 ± 5.2%. There was also no effect on the bradycardia and hypotension caused by vagal stimulation in the presence of citalopram. Hypotension was -37 ± 5 mmHg and bradycardia was -40 ± 3 bpm. This was compared to vehicle control, after which hypotension was -37 ± 4 mmHg and bradycardia was 48 ± 6 bpm.

Intravenous application of the mixed 5-HT and noradrenaline transporter inhibitor fluoxetine (1mg kg^{-1}; n= 5, figure 5.13) caused no significant change in the evoked increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) when compared to application of vehicle control. After administration of fluoxetine the change over pre-drug control stimulations was only 1.7 ± 5.6%. There was also no effect on the bradycardia and hypotension caused by vagal stimulation in the presence of fluoxetine. Hypotension was -31 ± 2 mmHg and bradycardia was -39 ± 6 bpm.

Intravenous application of the noradrenaline transporter inhibitor desipramine (1mg kg^{-1}; n= 5, figure 5.14) caused no significant change in the evoked increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) when compared to application of vehicle control. After administration of desipramine the change over pre-drug control stimulations was 8.8 ± 12.6%. There was also no effect on the bradycardia and hypotension caused by vagal stimulation in the presence of desipramine. Hypotension was -23 ± 6 mmHg and bradycardia was -52 ± 9 bpm.

Intravenous application of the OCT3 and PMAT inhibitor D-22 (600µg kg^{-1}; n= 5, figure 5.15) caused a significant potentiation (P<0.001) of the evoked increase in

extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) when compared to application of vehicle control (Figure 5.15). The first simulation subsequent to application of D-22 was increased 111 ± 21% over pre-drug control stimulations (Figure 5.14). D-22 also increased the bradycardia caused by vagal stimulation to 98 ± 11 bpm, which was significantly different from vehicle control (48 ± 6 bpm). Additionally, hypotension caused by vagal stimulation in the presence of D-22 was reduced to -9 ± 2 mmHg.

Combined data is shown in figure 5.27.

Effect of 5-HT_{1A} receptor blockade and re-uptake inhibition

Intravenous application of the 5-HT_{1A} receptor antagonist WAY-100635 (1mg kg^{-1}; n= 4, figure 5.18A) caused no significant change in the expected increase in extracellular 5-HT concentration in the NTS following vagal stimulation (20Hz, 10x threshold for 10s) when compared to application of vehicle control. After administration of WAY-100635 the change over pre-drug control stimulations was -5 ± 9.3%. In the presence of citalopram (1mg kg^{-1}; n=5, figure 5.18B) the change was -3 ± 5%. Both were not significantly different from vehicle control.

Injection of WAY-100635 alone and in the presence of citalopram was associated with a small decrease in baseline blood pressure and an increase in heart rate. This was not seen when it was administered in the presence of citalopram (see table 5.1). Alone, WAY-100635 decreased the bradycardia caused by vagal stimulation to -20 ± 1bpm, which was significantly different from vehicle control (46 ± 5 bpm). Additionally, hypotension caused by vagal stimulation in the presence of WAY-100635 was also reduced to -17 ± 4 mmHg, which was significantly different from vehicle control (36 ± 6 mmHg). Interestingly, this was reversed when WAY-100635 was administered in the presence of citalopram. Bradycardia caused by vagal stimulation was 48 ± 6 bpm and hypotension was 34 ± 6 mmHg, both not significantly different from control. Combined data is shown in figure 5.19.

Figure 5.12 Vagal stimulation trace: Citalopram

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of citalopram (1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 10s at 10X threshold) of the central end of the left vagus.

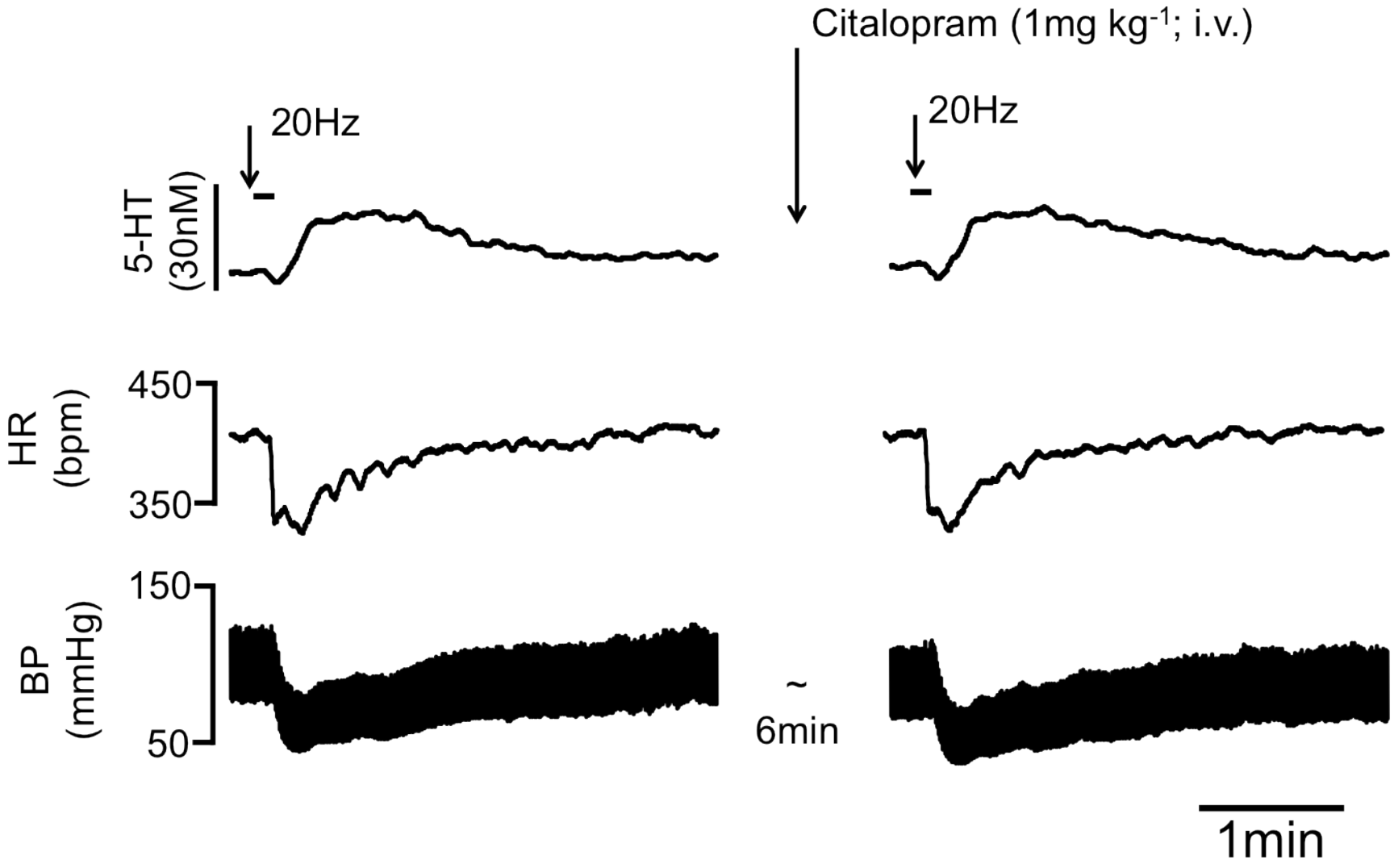

Citalopram (1mg kg-1; i.v.)
20Hz
20Hz
5-HT
(30nM)
HR
(bpm)
450
350
BP
(mmHg)
150
50
~
6min
1min

Figure 5.13 Vagal stimulation trace: Fluoxetine

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of fluoxetine (1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus.

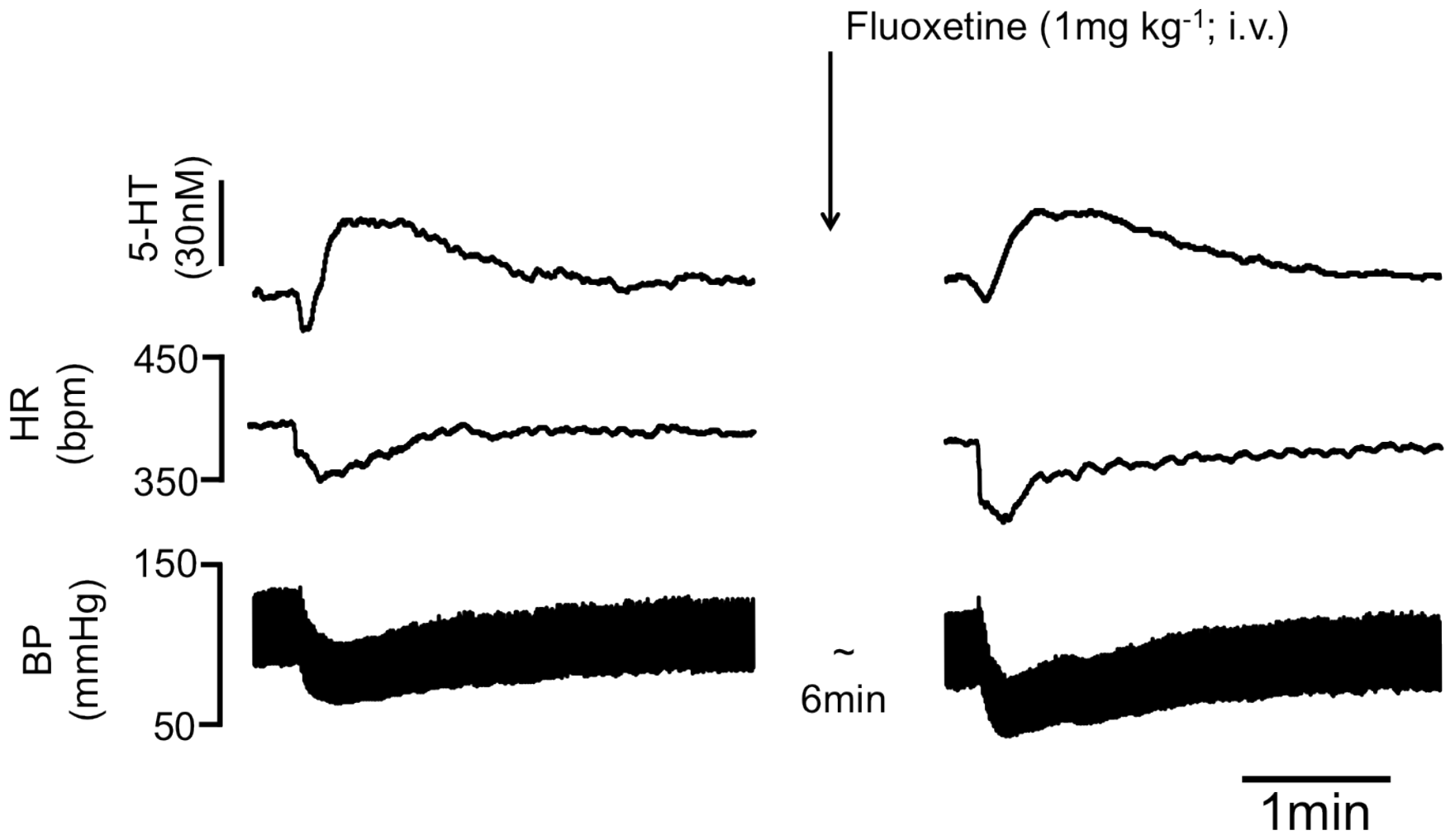

Fluoxetine (1mg kg-1; i.v.)
5-HT
(30nM)
HR
(bpm)
450
350
BP
(mmHg)
150
50
~
6min
1min

Figure 5.14 Vagal stimulation trace: Desipramine

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of desipramine (1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus.

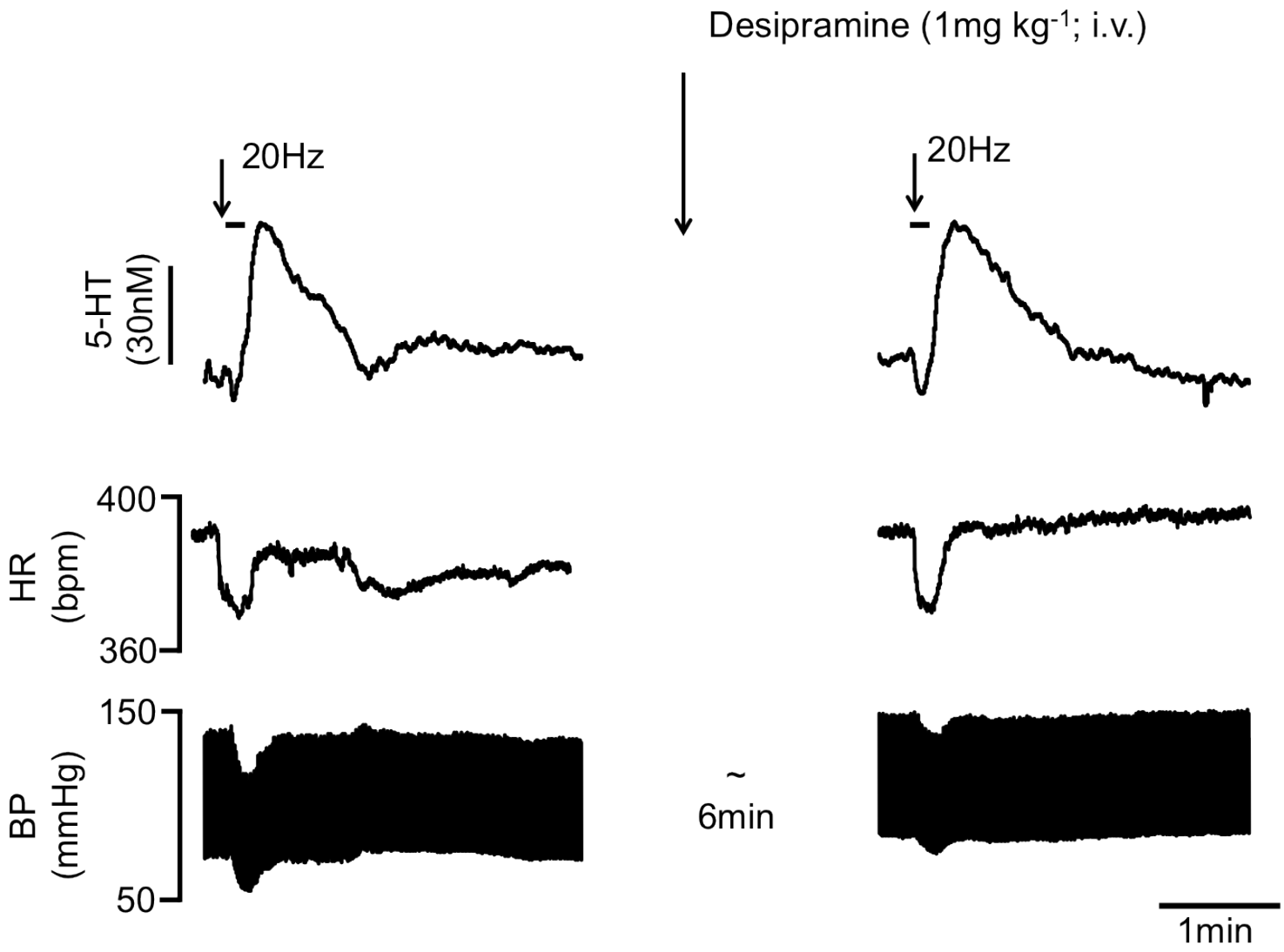

Desipramine (1mg kg-1; i.v.)
20Hz
20Hz
5-HT
(30nM)
400
HR
(bpm)
360
150
BP
(mmHg)
50
~
6min
1min

Figure 5.15 Vagal stimulation trace: D-22

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of decynium-22 (D-22; 1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus.

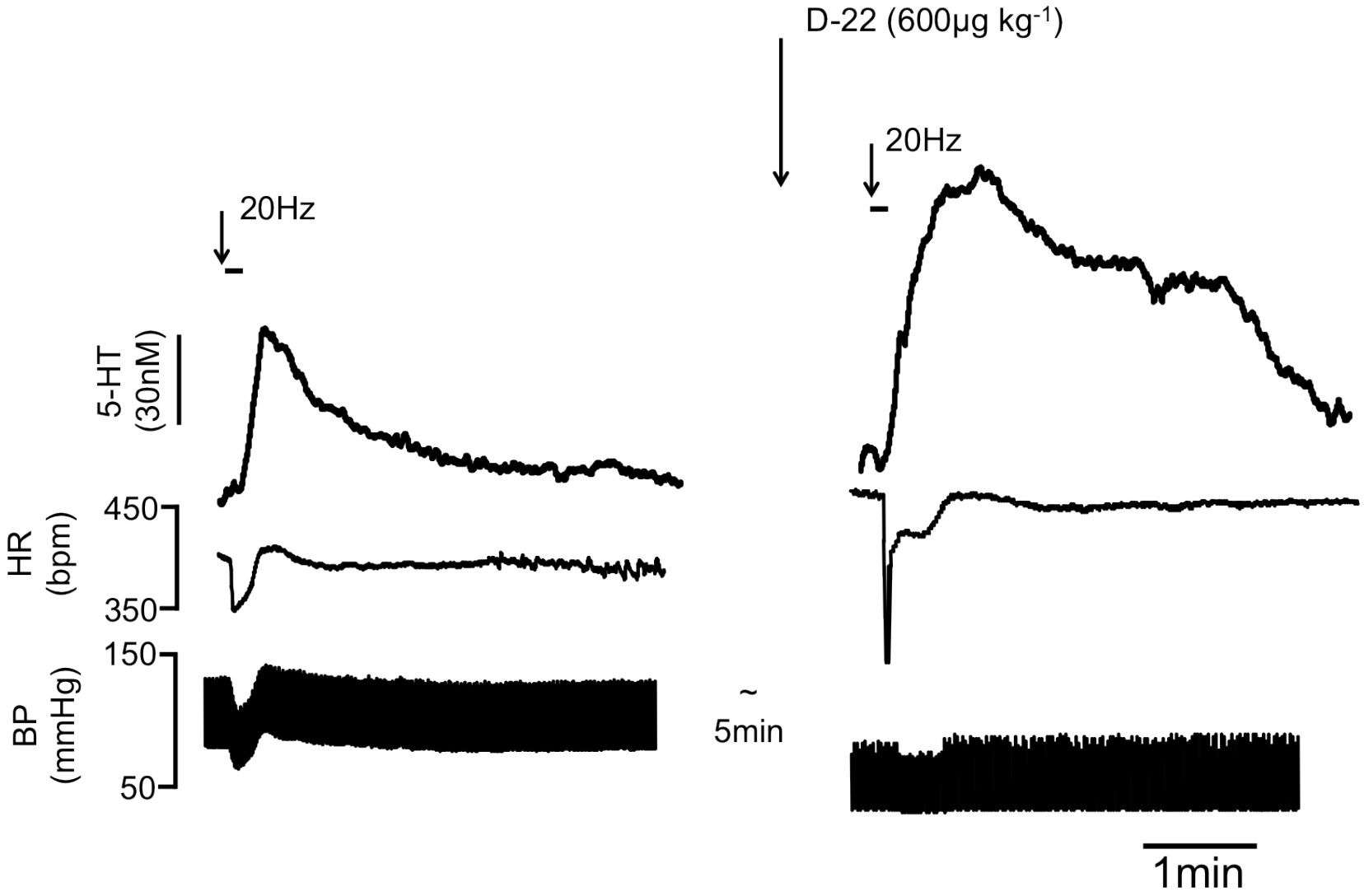

D-22 (600μg kg-1)
20Hz
20Hz
5-HT
(30nM)
HR
(bpm)
450
350
BP
(mmHg)
150
50
~
5min
1min

Figure 5.16 Vagal stimulation trace: Chlorisondamine

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the effect of chlorisondamine (1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus.

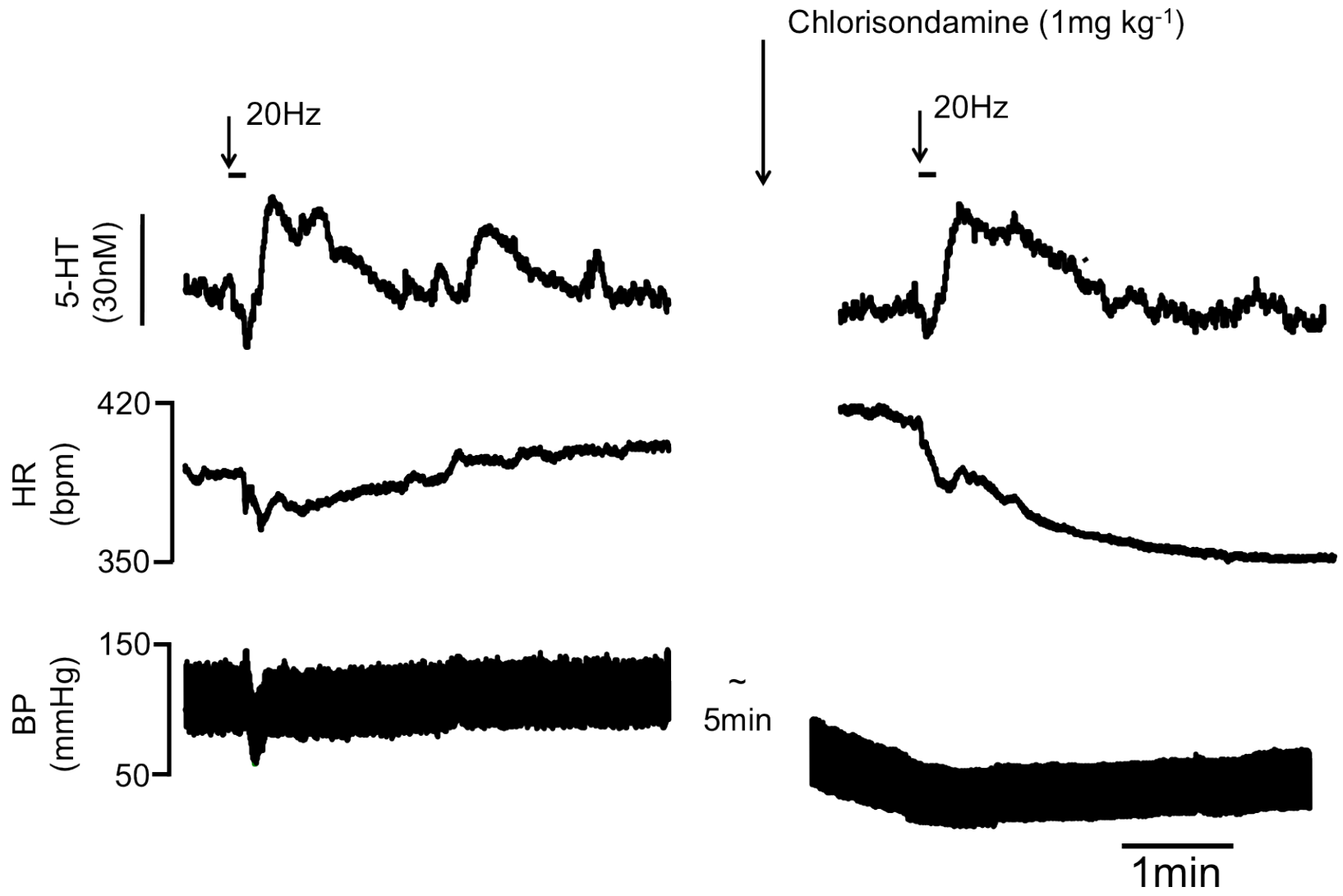

Chlorisondamine (1mg kg-1)
20Hz
20Hz
5-HT
(30nM)
HR
(bpm)
420
350
BP
(mmHg)
150
50
~
5min
1min

Figure 5.17 Comparison of the effect of fluoxetine, citalopram, cdecynium-22 and desipramine (re-uptake inhibitors): Combined data

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of i.v. saline (control; 1ml kg^{-1}, n=6), fluoxetine (1 mg kg^{-1}, n=5), citalopram (1mg kg^{-1}, n=6), decynium-22 (D-22; 600μg kg^{-1}, n=6) and desipramine (1mg kg^{-1}, n=6) on increases in the 5-HT concentration in the NTS and the decreases in mean arterial blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus. The bars show the s.e.m. These changes were compared to control using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. ** $P<0.01$; *** $P<0.01$.

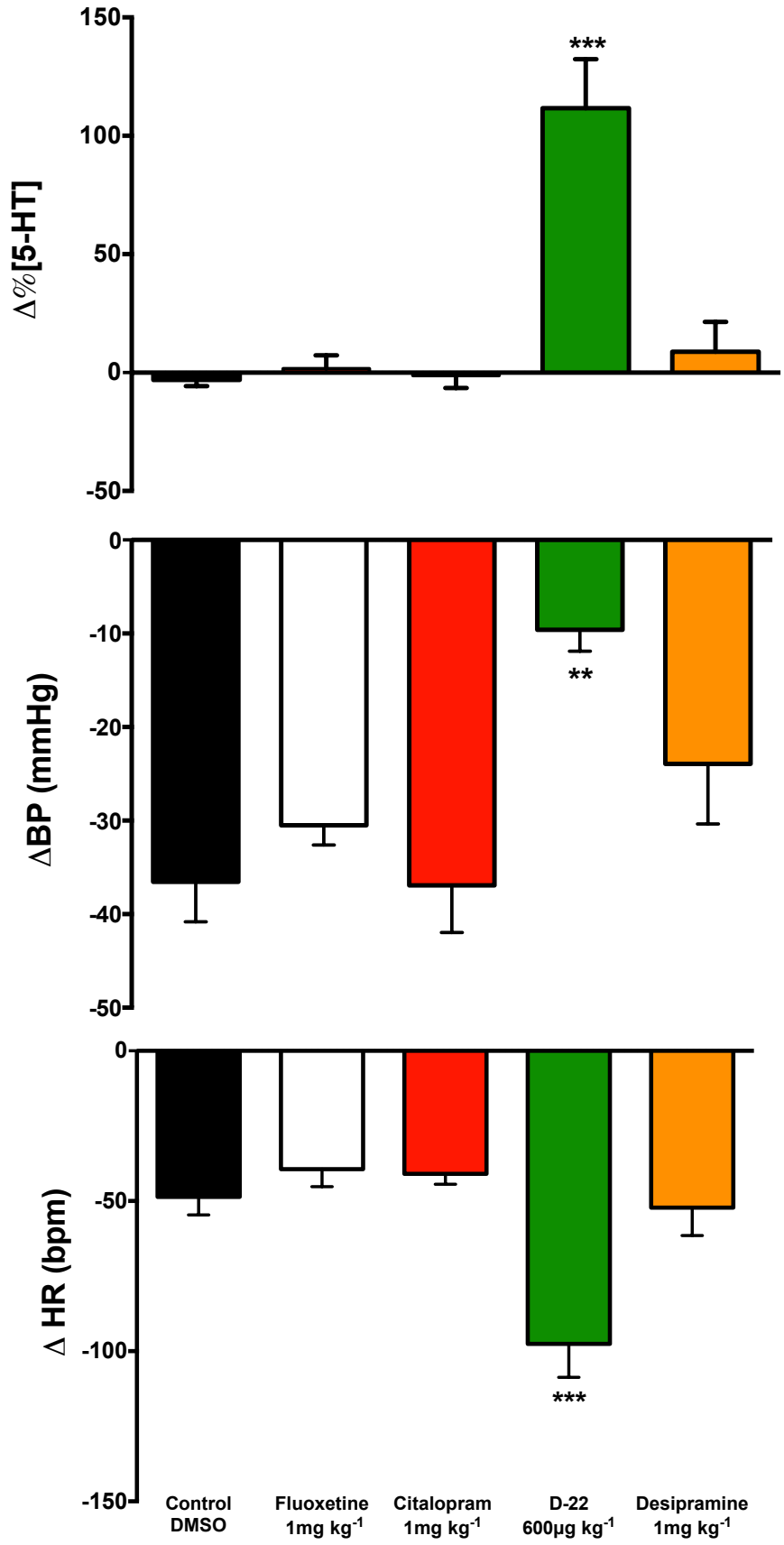
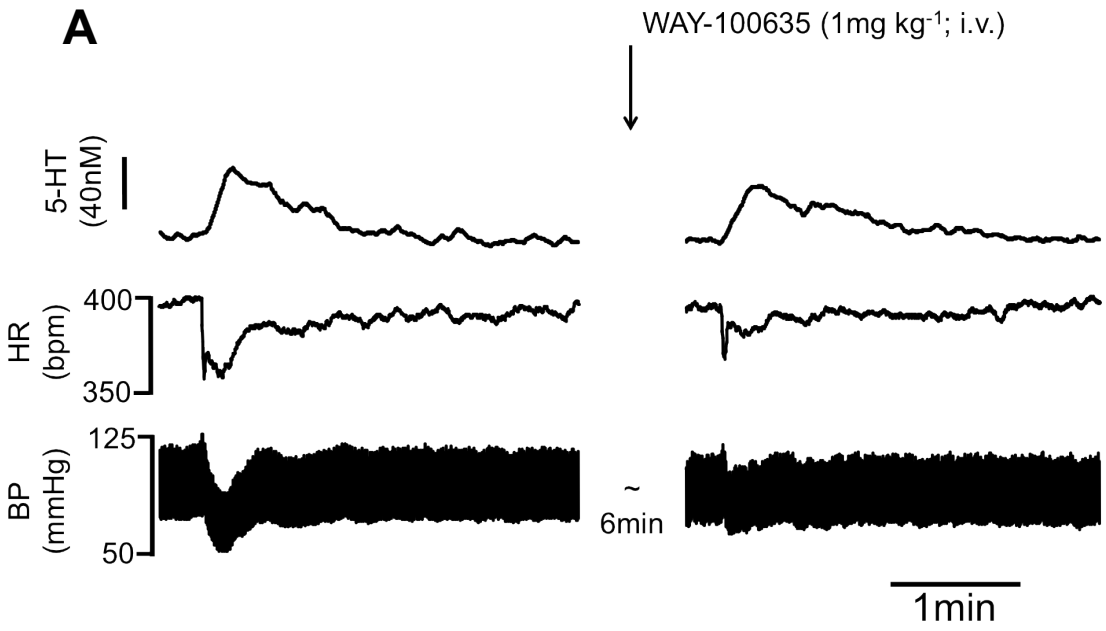

150
100
50
0
-50
Δ%[5-HT]
***
0
-10
-20
-30
-40
-50
ΔBP (mmHg)
**
0
-50
-100
-150
Δ HR (bpm)
***
Control
DMSO
Fluoxetine
1mg kg-1
Citalopram
1mg kg-1
D-22
600µg kg-1
Desipramine
1mg kg-1

Table 5.1 Baseline values: re-uptake inhibitor experiments

Test	Change (Δ) from baseline caused by test		Baseline values		
	Δ BP (mmHg)	ΔHR (bpm)	Evoked 5-HT (nM)	BP (mmHg)	HR (bpm)
Vehicle DMSO (100µl kg^{-1})	2 ±5	5 ± 8	25 ± 9	90 ± 6	390 ± 9
Fluoxetine (1mg kg^{-1})	15 ± 5	20 ± 4	33 ± 8	115 ± 18	410 ± 15
Citalopram (1mg kg^{-1})	10 ± 2	12 ± 5	28 ± 4	105 ± 8	388 ± 10
D-22 (600µg kg^{-1})	-49 ± 7	86 ± 15	29 ± 10	97 ± 4	399 ± 6
Desipramine (1mg kg^{-1})	20 ± 4	15 ± 4	38 ± 5	89 ± 4	380 ± 4
WAY-100635 (1mg kg^{-1})	-12 ± 5	11 ± 4	28 ± 3	96 ± 7	389 ± 10
WAY-100635 (1mg kg^{-1}) + Citalopram (1mg kg^{-1})	-15 ± 5	18 ± 8	23 ± 8	110 ± 3	415 ± 12

Figure 5.18 Effect of WAY-100635 alone and in the presence of Citalopram: Experimental Traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rats showing the effect of A) WAY-100635 (1 mg kg^{-1}; i.v.) alone and B) in the presence of citalopram (1 mg kg^{-1}; i.v.) on the increase in the 5-HT concentration in the NTS and the decreases in blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus.


A
WAY-100635 (1mg kg-1; i.v.)
5-HT (40nM)
HR (bpm)
400
350
BP (mmHg)
125
50
~ 6min
1min

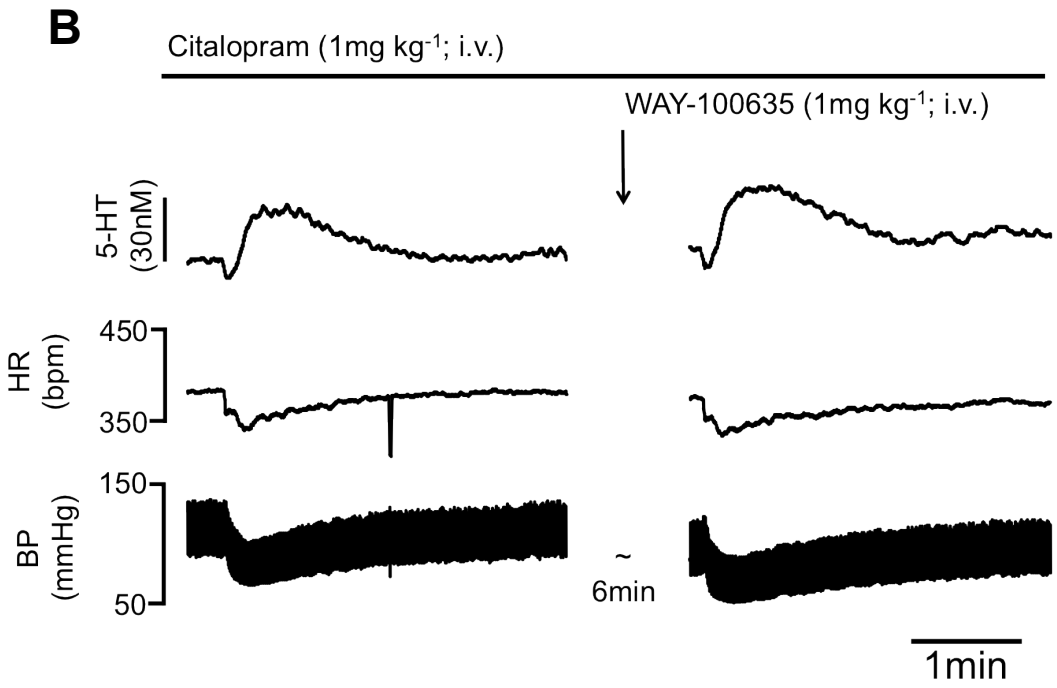
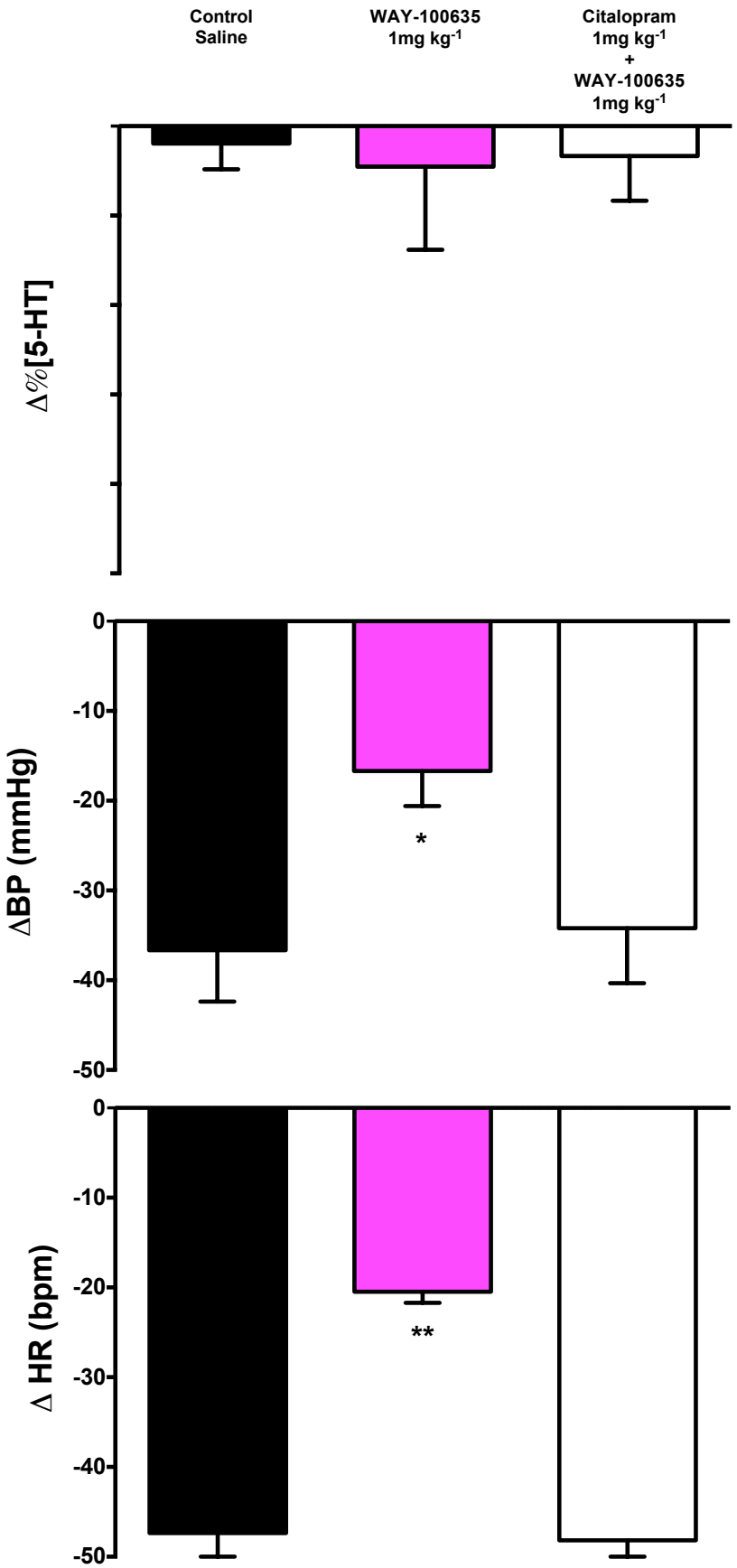

B
Citalopram (1mg kg-1; i.v.)
WAY-100635 (1mg kg-1; i.v.)
5-HT (30nM)
HR (bpm)
450
350
BP (mmHg)
150
50
~ 6min
1min

Figure 5.19 Effect of WAY-100635 alone and in the presence of citalopram: Combined data

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects (i.v.) of saline (control, n=5), WAY-100635 (1 mg kg^{-1}, n=4) alone and in the presence of citalopram (1mg kg^{-1}, n=5) on increases in the 5-HT concentration in the NTS and the decreases in mean arterial blood pressure (BP) and heart rate (HR) evoked by electrical stimulation (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus. The bars show the s.e.m. These changes were compared to control using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. * $P<0.05$

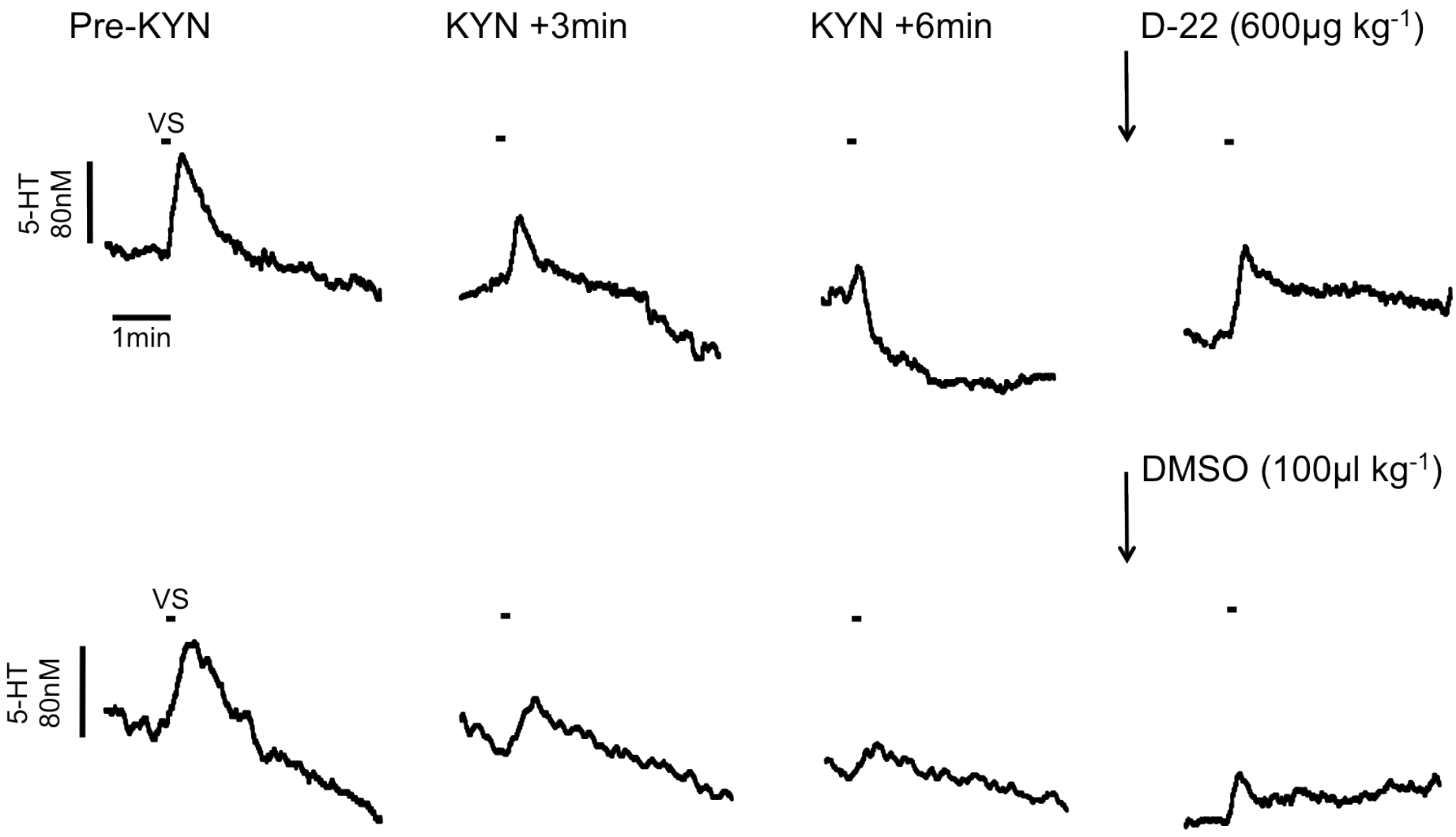

Control
Saline
WAY-100635
1mg kg-1
Citalopram
1mg kg-1
+
WAY-100635
1mg kg-1
Δ%[5-HT]
ΔBP (mmHg)
0
-10
-20
-30
-40
-50
*
Δ HR (bpm)
0
-10
-20
-30
-40
-50
**

5.9. Results: D-22 and KYN

Kynurenate (KYN; 10mM) was applied topically to the brainstem, as detailed in section 5.6. Prior to application of KYN the extracellular increase in 5-HT produced by vagal stimulation (20Hz, 10x threshold) changed by 5 ± 6% over pre-DMSO vehicle controls (n=5, figure 5.20) in the vehicle group and -6 ± 7% in the D-22 group (n=5, figure 5.20). 3min post application of KYN extracellular 5-HT increases were reduced by 41 ± 6% in the vehicle group and -35 ± 9% in the D-22 group. 6min post application of KYN extracellular 5-HT increases were reduced by 60 ± 3% in the vehicle group and -57 ± 6% in the D-22 group. This was significantly different ($P<$ 0.001) from the extracellular increase in 5-HT pre-KYN application. There were, however, no significant differences between the vehicle control group and D-22 group, at this stage. Subsequently in the presence of D-22 (600µg kg^{-1}), extracellular increases in 5-HT produced by vagal stimulation returned to that observed pre-KYN now only changing 5 ± 17 % from pre-KYN control stimulations. This was significantly different ($P<$ 0.001) from DMSO (100µl kg^{-1}) vehicle control that was still reduced by 53 ± 5% of pre-KYN control stimulations (figure 5.21).

Figure 5.20 Effect of decynium-22 (D-22) in the presence of Kynurenate: Experimental Traces

Two anaesthetised artificially ventilated, neuromuscular-blocked male rats showing the effect of topically applied kynurenic acid (30 µl, 10 mM) on the increase in the 5-HT concentration in the NTS evoked by a series of electrical stimulations (20Hz, 1ms, 3s at 10X threshold) of the central end of the left vagus. Stimulations were applied at 3 min intervals as shown by the line (VS) above the traces. Also shown is the effect of decynium-22 (D-22; 600 µg kg-1, top set of traces) or vehicle (DMSO, 100 µl kg-1; bottom set of traces) 6min after application of KYN.


Pre-KYN
KYN +3min
KYN +6min
D-22 (600µg kg-1)
VS
5-HT
80nM
1min
DMSO (100µl kg-1)
VS
5-HT
80nM

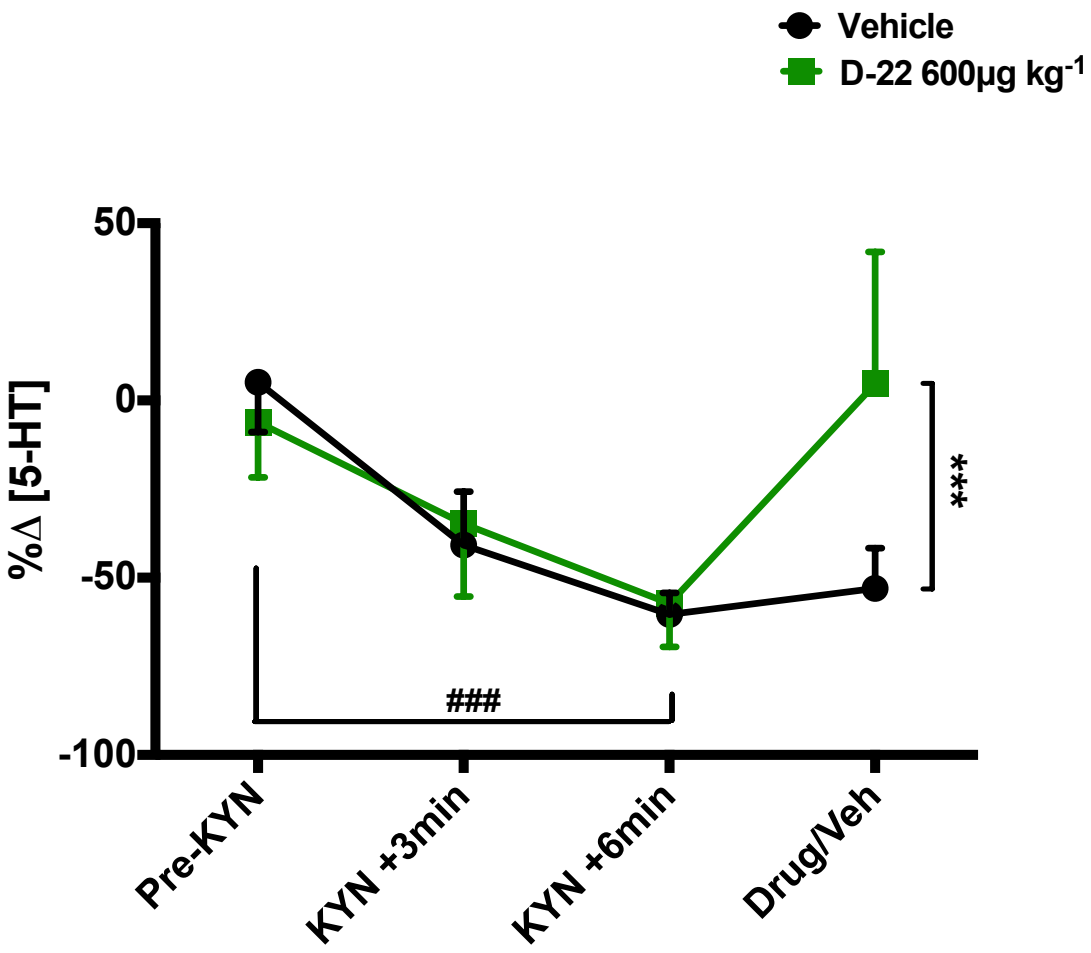


Figure 5.21 Effect of kynurenate and D-22: Graph

Percentage mean (±s.e.m) peak changes over control in extracellular 5-HT concentration recorded by voltammetry in the NTS following electrical vagal stimulation (20Hz, 10x threshold) before (Pre-KYN) topical application of 10mM kynurenate, 3 min and 6 min post-application (KYN +3min and KYN, +6min, respectively). After 6min decynium-22 (D-22; 600µg kg^{-1}) or vehicle (DMSO; 100µl kg^{-1}) is applied i.v.

*** $P<0.01$ 2-way ANOVA; compared to vehicle with Bonferroni's test.

$P<0.01$ 2-way ANOVA; compared to pre-KYN with Bonferroni's test for both vehicle and D-22 experiments.

5.10. Results: Cardiovascular reflex activation

Cardiopulmonary reflex

Activation of the cardiopulmonary reflex by intra atrial application of the 5-HT_3 agonist phenybiguanide (PBG; 3µg per animal, n=4) caused an increase in extracellular 5-HT of 7±1.5nM, 10µg per animal (n=4) caused an increase of 23 ± 1.6nM and 30µg per animal (n=5, figure 5.22) caused an increase of 46 ± 5.2nM. Effects caused by 10 and 30µg per animal were significantly different ($P< 0.001$) from changes measured over the same time period after intra atrial application of saline (100µl per animal), -1.3 ± 2nM. Mean changes over baseline are shown in figure 5.26.

Chemoreflex reflex

Activation of the chemoreflex by i.v application of the sodium cyanide (NaCN; 50µg per animal, n=3) caused a increase in extracellular 5-HT of 13.0 ± 3.5nM, 100µg per animal (n=3) caused a change of 40.4 ± 10.6nM and 200µg per animal (n=3, figure 5.23) caused a change of 53.5.2 ± 9.3nM. Changes in extracellular 5-HT caused by 100 and 200µg per animal were significantly different ($P< 0.001$) from changes measured over the same time period after intra atrial application of saline (100µl per animal), -1.3 ± 2nM. Mean changes over baseline are shown in figure 5.27.

Depressor reflex

Activation of the depressor reflex by i.v. application of noradrenaline (NA; 0.5µg per animal, n=3) caused a change in extracellular 5-HT of 0.7 ± 1.7nM, 10µg per animal (n=4) caused a change of 11.6 ± 5.7nM and 50µg per animal (n=4, figure 5.24) caused a change of 23.2 ± 11.5nM. Effects caused by 50µg per animal were significantly different ($P< 0.05$) from changes measured over the same time period after intra atrial

application of saline (100µl per animal), -1.3 ± 2nM. Mean changes over baseline are shown in figure 5.28.

Pressor reflex

Activation of the pressor reflex by i.v. application of sodium nitroprusside (SNP; 5µg per animal, n=4) caused a change in extracellular 5-HT of 1 ± 4.0nM, 10µg per animal (n=4) caused a change of 3.5 ± 2.0nM and 50µg per animal (n=4, figure 5.22) caused a change of 3.2 ± 5.4nM. These effects were not significantly different from changes measured over the same time period after intra atrial application of saline (100µl per animal), -1.3 ± 2nM. Mean changes over baseline are shown in figure 5.29.

Figure 5.22 Cardiopulmonary reflex: Traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the increase in the 5-HT concentration in the NTS and decreases in arterial blood pressure (BP) and heart rate (HR) caused by stimulation of the cardiopulmonary reflex by intra-atrial (i.a) injection of phenylbiguanide (PBG; 30 μg in 10 μl).

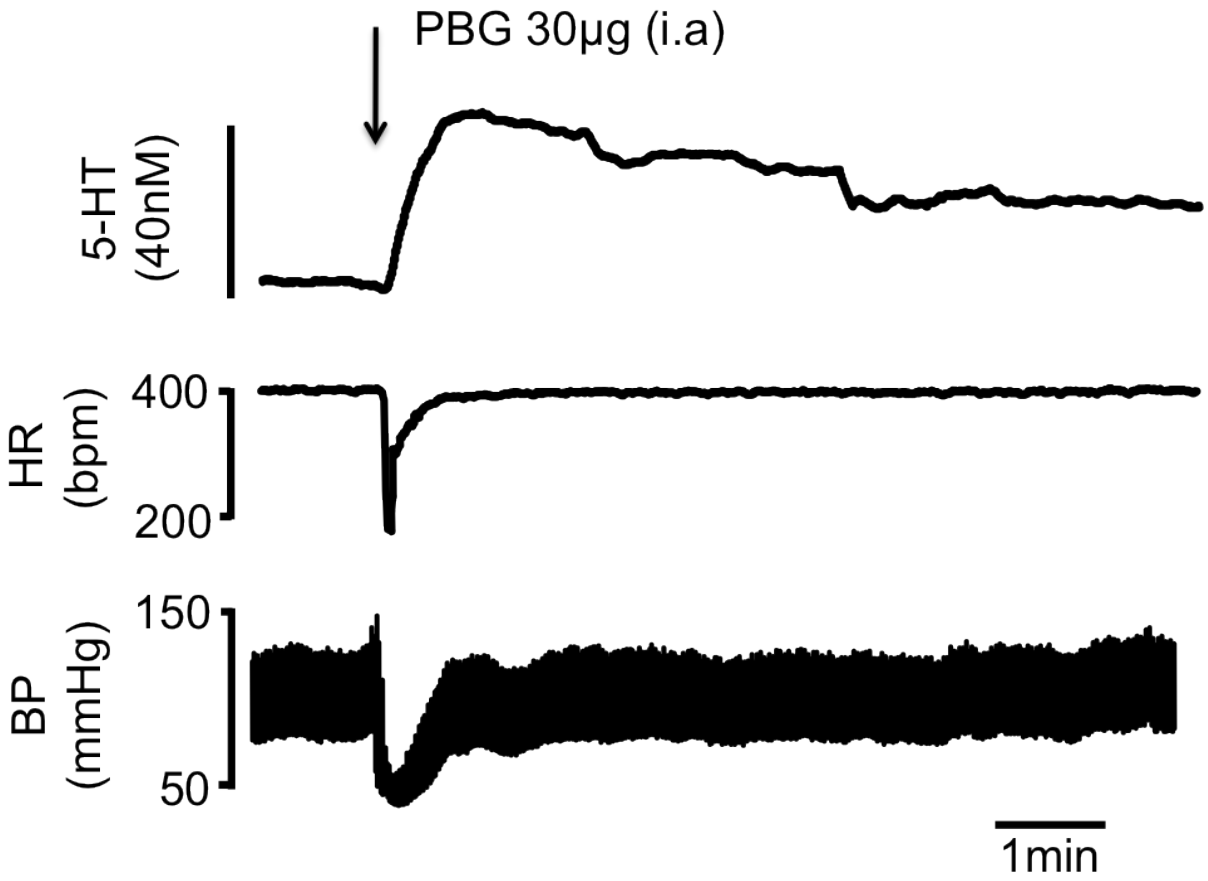

PBG 30μg (i.a)
5-HT
(40nM)
HR
(bpm)
400
200
BP
(mmHg)
150
50
1min

Figure 5.23 Chemoreflex: Traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the increase in the 5-HT concentration in the NTS and decrease in arterial blood pressure (BP) and biphasic changes in heart rate (HR) caused by stimulation of the chemoreflex reflex by intra atrial (i.a) injection of sodium cyanide (NaCN; 200 μg in 10 μl).

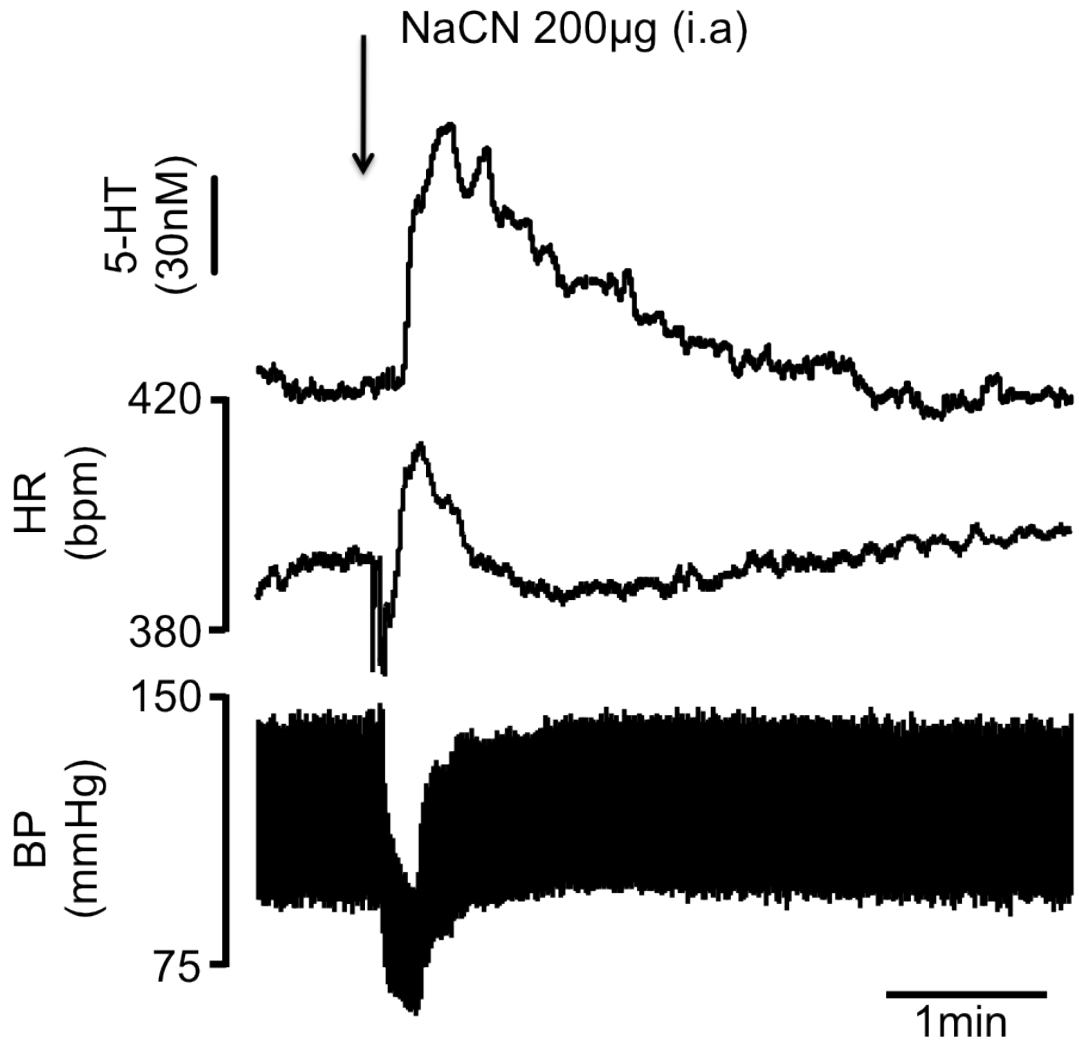

NaCN 200µg (i.a)
5-HT
(30nM)
420
HR
(bpm)
380
150
BP
(mmHg)
75
1min

Figure 5.24 Depressor reflex: Traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the increase in the 5-HT concentration in the NTS and increases in arterial blood pressure (BP) and decrease in heart rate (HR) caused by an i.v. injection of noradrenaline (NA; 50μg).

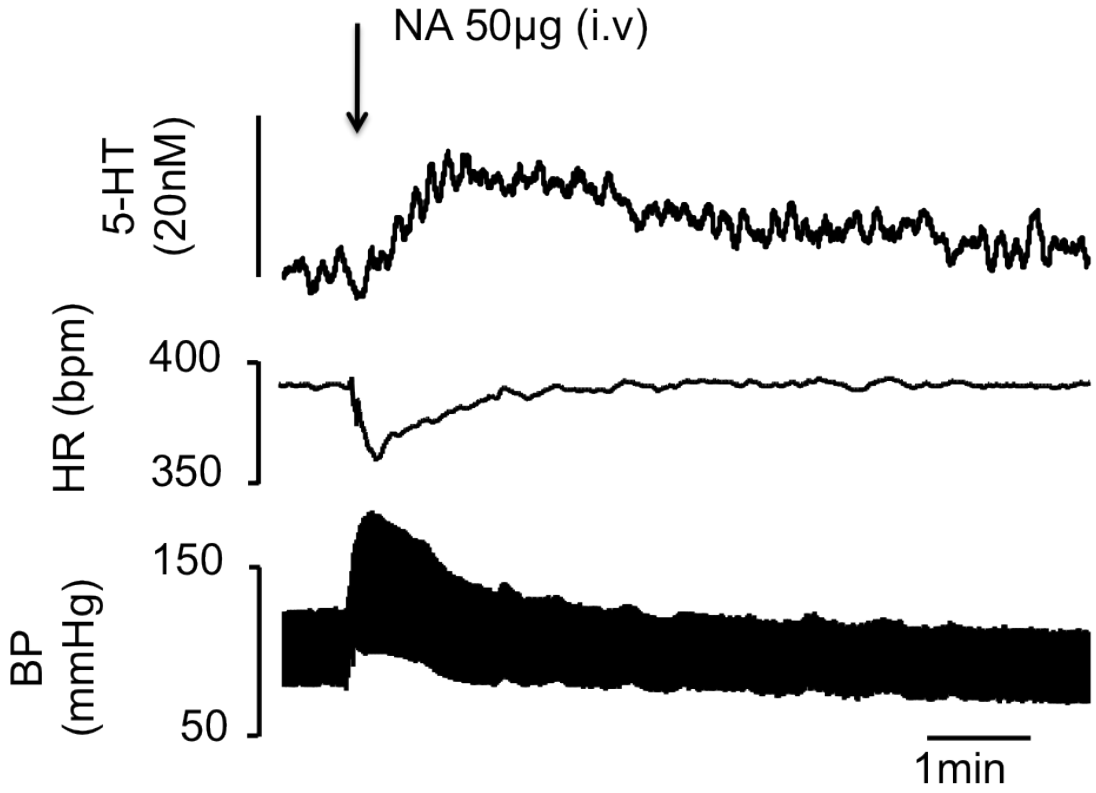
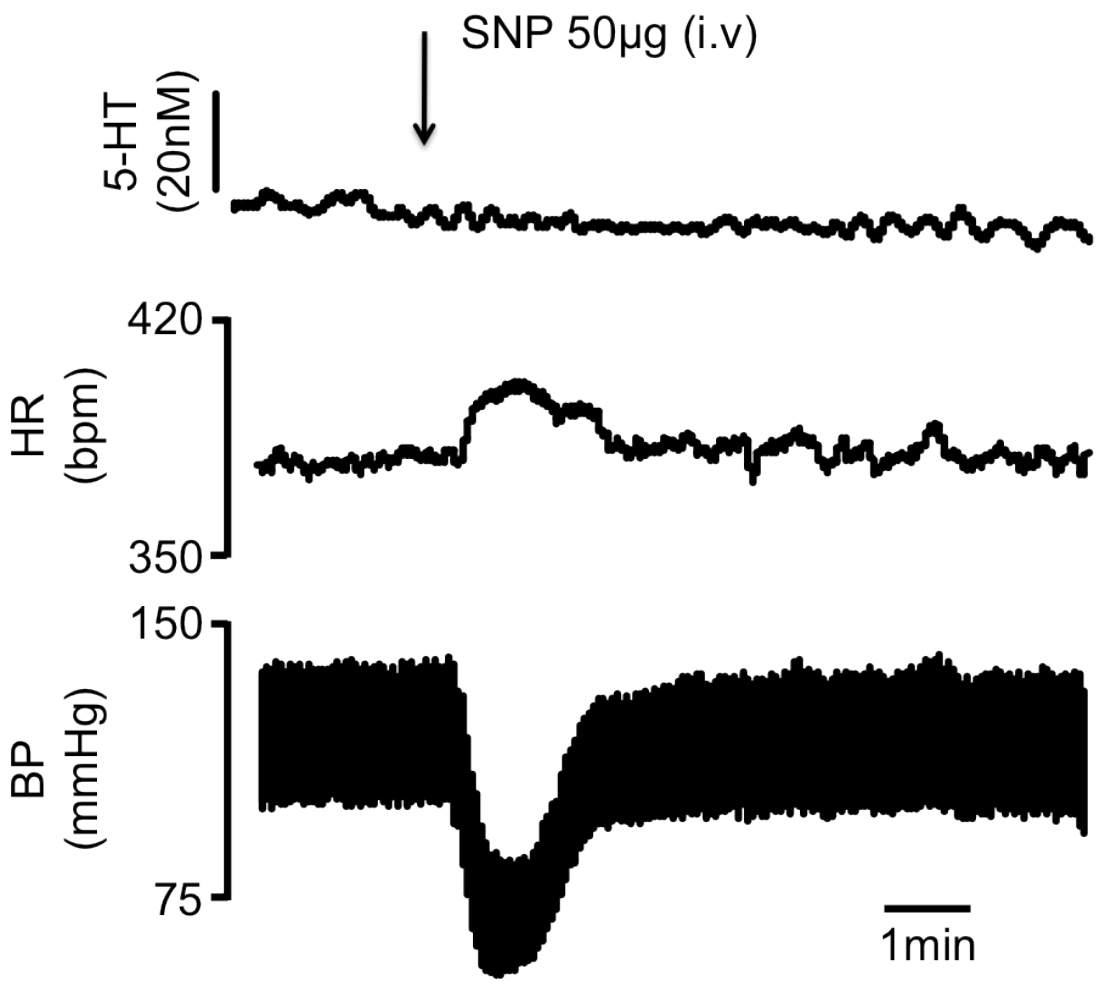

NA 50µg (i.v)
5-HT
(20nM)
HR (bpm)
400
350
BP
(mmHg)
150
50
1min

Figure 5.25 Pressor reflex: Traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing no change in the 5-HT concentration in the NTS, a decrease in arterial blood pressure (BP) and increase in heart rate (HR) caused by an i.v. injection of sodium nitroprusside (SNP; 50μg)


SNP 50μg (i.v)
5-HT
(20nM)
420
HR
(bpm)
350
150
BP
(mmHg)
75
1min

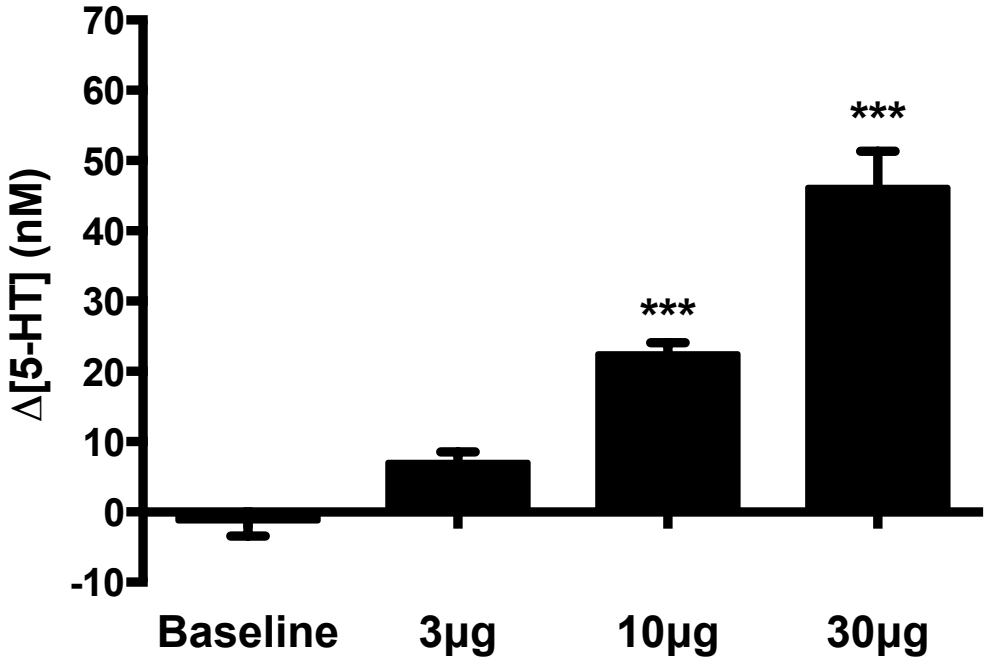


Figure 5.26 Cardiopulmonary reflex: Histogram

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of intra-atrial (i.a) injections (10 µl) of different doses (3, 10 & 30 µg; n = 5) of phenylbiguanide with that of saline on the 5-HT concentration in the NTS. The bars show the s.e.m. These changes were compared to saline using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. *** $P<0.001$

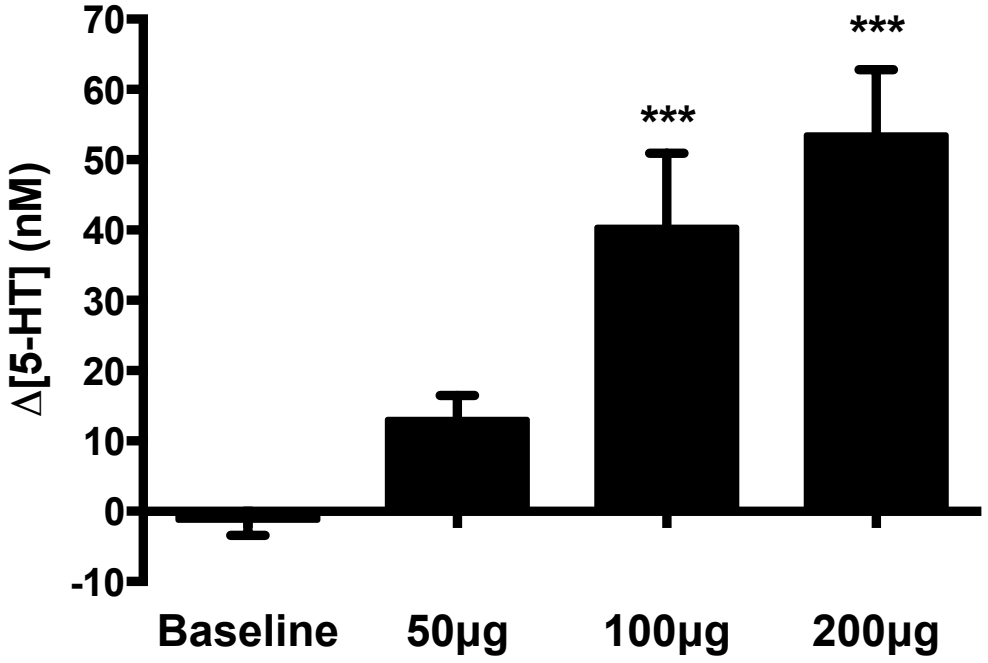


Figure 5.27 Chemoreflex: Histogram

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of intra-atrial (i.a) injections (10 µl) of different doses (50, 100 & 200 µg; n = 3) of sodium cyanide on 5-HT concentration in the NTS. The bars show the s.e.m. These changes were compared to saline using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. *** P<0.001

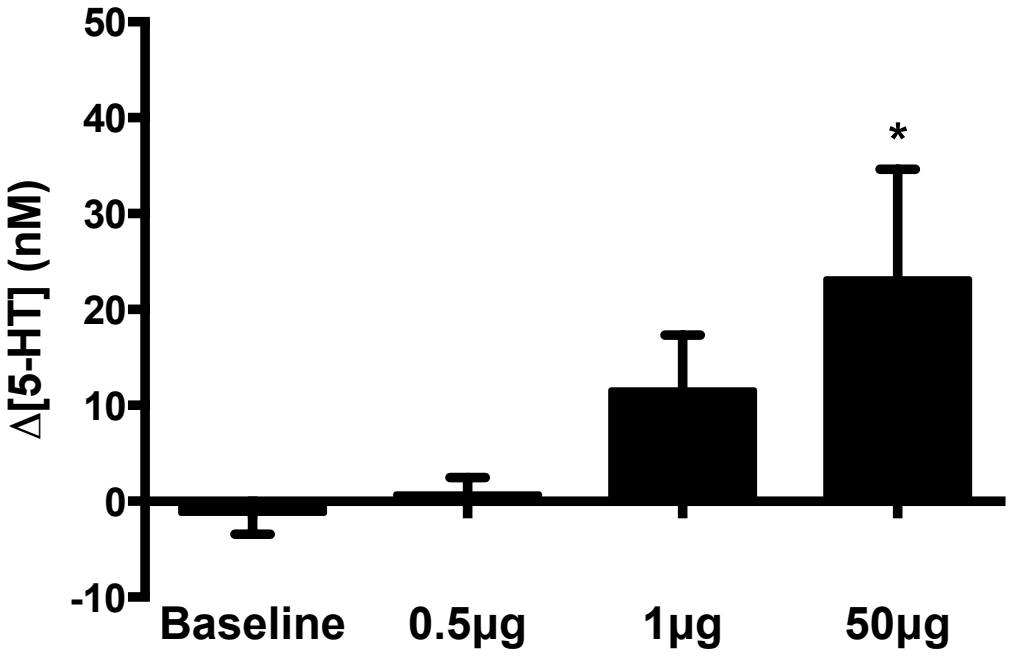


Figure 5.28 Depressor reflex: Histogram

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of i.v. injections of different doses (0.5, 1 & 50 µg; n = 4) of noradrenaline on the 5-HT concentration in the NTS. The bars show the s.e.m. These changes were compared to saline using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. * $P<0.05$.

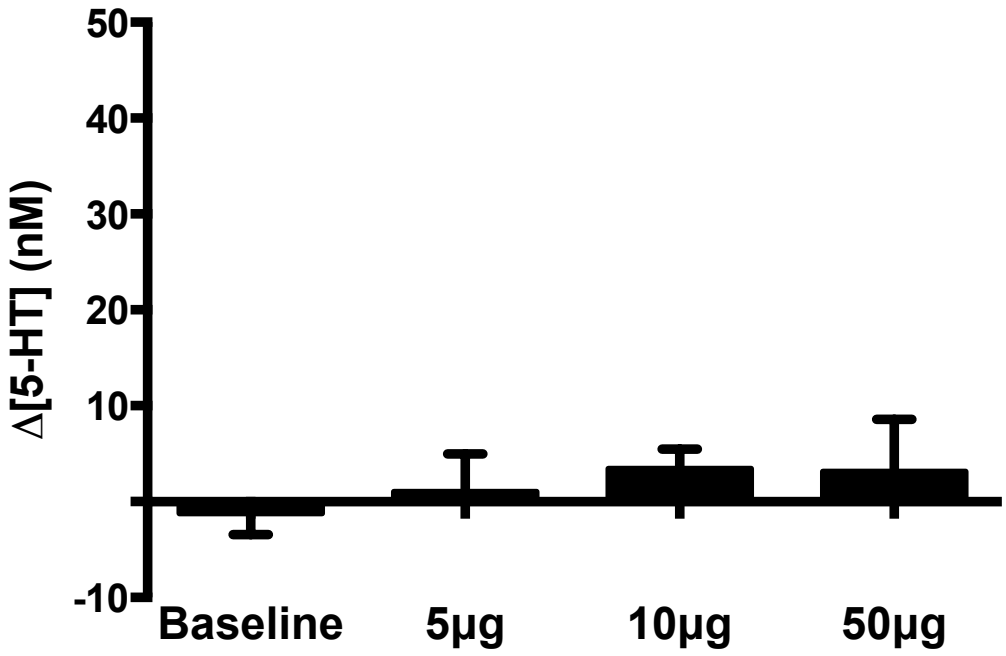


Figure 5.29 Pressor reflex: Histogram

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects of i.v. injections of different doses (5, 10 & 20 µg; n = 4) of sodium ntroprusside with that of saline on the 5-HT concentration in the NTS. The bars show the s.e.m. These changes were compared to saline using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test and were found to be not significant.

5.11. Results: Effect of re-uptake inhibitors on the cardiopulmonary reflex

Hemodynamic baseline data

Baseline heart rate and blood pressure were measured 30s prior to commencement of the experimental protocol. The vehicle control experimental group had a baseline blood pressure of 112 ± 3 mmHg and a baseline heart rate of 410 ± 5 bpm. The citalopram experimental group had a baseline blood pressure of 102 ± 4 mmHg and a baseline heart rate of 399 ± 4 bpm. The D-22 experimental group had a baseline blood pressure of 105 ± 6 mmHg and a baseline heart rate of 401 ± 5 bpm.

5-HT release baseline data

The average absolute increases of extracellular 5-HT measured in response to phenylbiguanide (PBG; 10μg per animal) were 25 ± 6nM in the vehicle control experimental group, 18 ± 5nM in the citalopram group and 22 ± 8nM in the D-22 group.

Effect of re-uptake inhibitors

Intravenous application of the selective 5-HT transporter inhibitor citalopram (1mg kg^{-1}; n= 5, figure 5.30) caused no significant change in the evoked increase in extracellular 5-HT concentration in the NTS following intra atrial application of the 5-HT_3 agonist PBG. In the presence of citalopram the increase in extracellular 5-HT changed by -26.4 ± 12.4%. This was not significantly different from vehicle control (saline, 1ml kg^{-1}; n=6) control, which caused a change of -9.2 ± 7.6% over pre-drug controls. There were also no effects on the bradycardia and hypotension caused by PBG in the presence of citalopram, -60 ± 7 mmHg and -243 ± 40 bpm, respectively. This was compared to vehicle control, after which hypotension was -64 ± 10 mmHg and bradycardia was -141 ± 28 bpm.

Intravenous application of the OCT3 and PMAT inhibitor D-22 (600µg kg^{-1}; n= 5, figure 5.31) caused a potentiation of the expected increase in extracellular 5-HT concentration in the NTS following intra atrial application of the 5-HT_3 agonist PBG. In the presence of D-22 the increase in extracellular 5-HT was potentiated by -210 ± 53%. This was significantly different (P<0.001) compared from control (saline, 1ml kg^{-1}) control, which caused a change of -9.2 ± 7.6%. D-22 also increased the bradycardia caused by cardiopulmonary reflex activation, which now was -348 ± 32 bpm. This was significantly different from control. The hypotension caused by cardiopulmonary reflex activation was now 37 ± 7mmHg but was not significantly from control. Combined data is shown in figure 5.32.

Figure 5.30 Cardiopulmonary reflex traces: Citalopram

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the increase in the 5-HT concentration in the NTS and decreases in arterial blood pressure (BP) and heart rate (HR) caused by stimulation of the cardiopulmonary reflex by intra-atrial (i.a) injection of phenylbiguanide (PBG; 10 μg in 10 μl) in absence and presence of citalopram (1 mg kg^{-1}; i.v.). The 2nd PBG injection is 6 min after administration of citalopram.

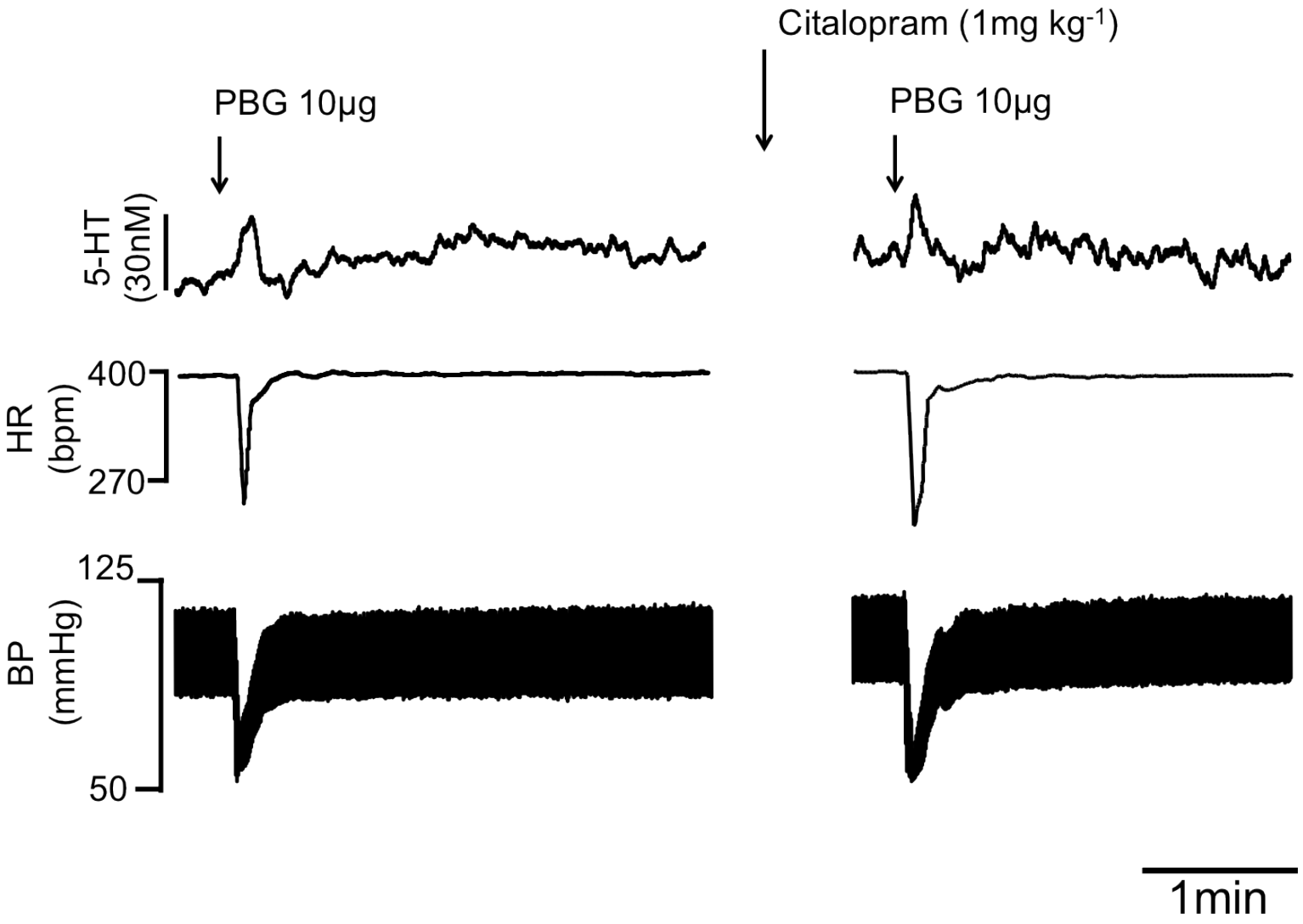

Citalopram (1mg kg-1)
PBG 10µg
PBG 10µg
5-HT
(30nM)
HR
(bpm)
400
270
BP
(mmHg)
125
50
1min

Figure 5.31 Cardiopulmonary reflex traces: D-22

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing the increase in the 5-HT concentration in the NTS and decreases in arterial blood pressure (BP) and heart rate (HR) caused by stimulation of the cardiopulmonary reflex by intra-atrial (i.a) injection of phenylbiguanide (PBG; 10 μg in 10 μl) in the absence and presence of decynium-22 (D-22; i.v., 600μg kg^{-1}). The 2^{nd} PBG injection is 3 min after administration of D-22.

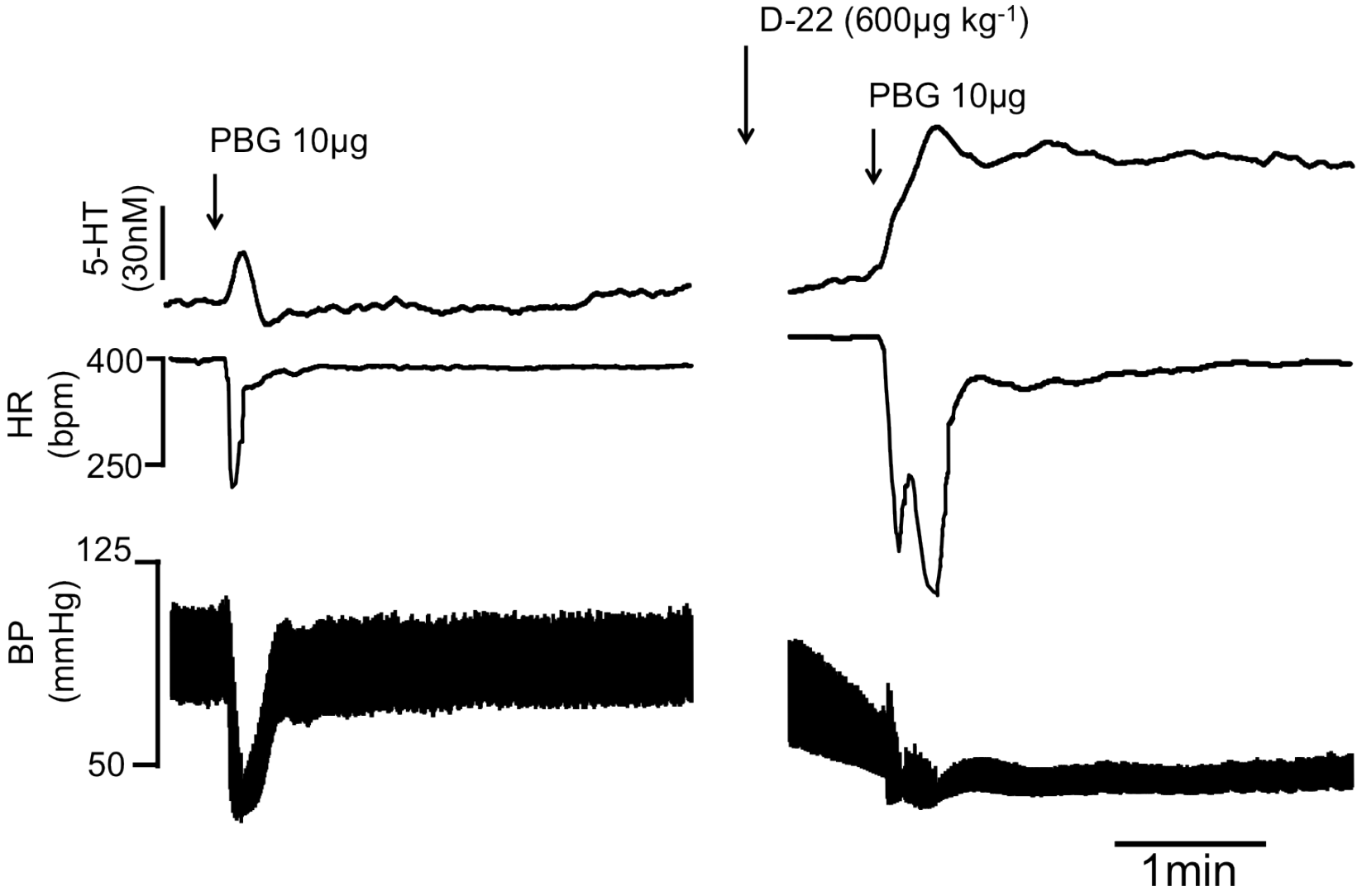
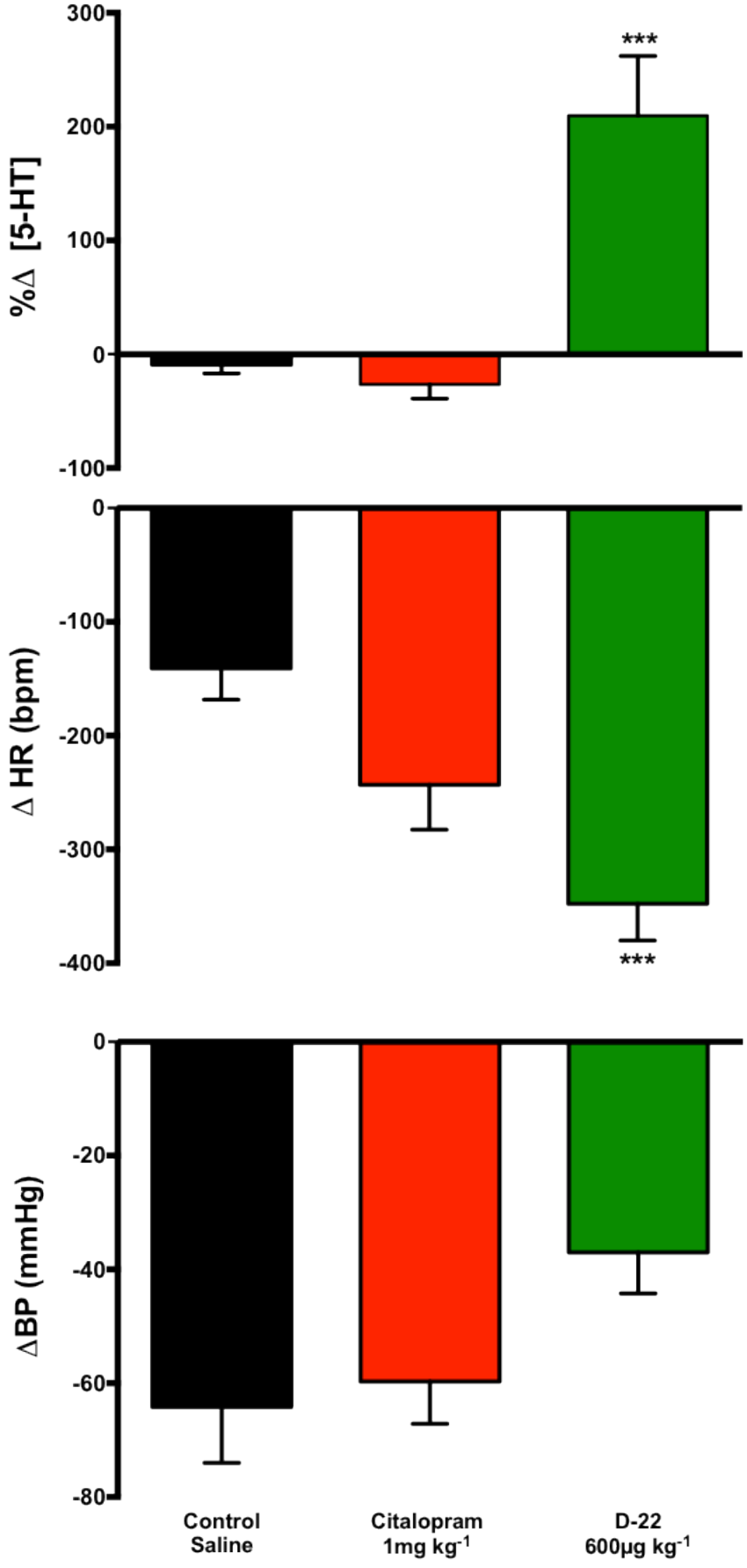

D-22 (600µg kg-1)
PBG 10µg
PBG 10µg
5-HT (30nM)
HR (bpm)
400
250
BP (mmHg)
125
50
1min

Figure 5.32 Effect of Citalopram and Decynium-22 on the cardiopulmonary reflex: Combined data

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects (i.v.) of saline (control, 1ml kg^{-1}, n=6), citalopram (1mg kg^{-1}, n=5) and decynium-22 (D-22; 600µg kg^{-1}, n=4). on increases in the 5-HT concentration in the NTS and the decreases in mean arterial blood pressure (BP) and heart rate (HR) caused by intra-atrial (i.a.) injection of phenylbiguanide (10 µg in 10 µl). The bars show the s.e.m. These changes were compared to control using a one-way analysis of variance (ANOVA) and the means compared with Fisher's LSD test. *** $P<0.001$


300
200
100
0
-100
%Δ [5-HT]
***
0
-100
-200
-300
-400
Δ HR (bpm)
***
0
-20
-40
-60
-80
ΔBP (mmHg)
Control
Saline
Citalopram
1mg kg-1
D-22
600µg kg-1

5.12. Discussion

5.12.1. Main findings

The present data demonstrate that activation of sensory afferents, either electrically or physiologically (cardiopulmonary, chemo- and baroreceptors), increases extracellular 5-HT in the NTS in anaesthetized rats. This 5-HT increase is stimulation frequency and calcium dependent, as detected by fast-cyclic voltammetry.

Further, the evidence indicates that the low affinity, high capacity re-uptake system consisting of OCT3 and/or PMAT is responsible for the removal of 5-HT released from vagal afferents.

5.12.2. Signal verification

Using the guidelines for validation of an electrochemical signal set out by Phillips and Wightman (2003) the present study first sought to first confirm the identity of the electrochemical signal recorded in the NTS. To this end, the *in vitro* data demonstrate a high selectivity for 5-HT over noradrenaline. Further, the electrochemical signal recorded *in vivo* closely matches that recorded *in vitro* with a known concentration of 5-HT. The release of detected 5-HT is also consistent with ability of 5−HT antagonist to block reflex activation of NTS neurons (Ramage & Villalon, 2008). However, the signal was not increased by blockade of 5-HT transporter (5-HTT/SERT) with citalopram (Hyttel, 1982) at a dose known to decrease the removal of 5-HT when directly applied to the NTS. Although the voltammetric scan is optimised for 5-HT, this observation may infer that the voltammetric electrode is detecting another monoamine. It is relevant here that noradrenaline terminals and cell bodies (the A2 group) are found in the NTS (Fuxe, 1965, Takahashi *et al.*, 1980, Kalia *et al.*, 1985, see Rinaman, 2011) and further vagal afferents also make synaptic connection with the A2 group (Sumal *et al.*, 1983) and can activate these neurons (Appleyard *et al.*, 2007). Nevertheless, in the present study the selective noradrenaline uptake inhibitor

desipramine (Glowinski & Axelrod, 1964), at a dose that decreases the removal of noradrenaline applied to NTS but not 5-HT, also failed to increase the evoked voltammetry signal. Furthermore, the evoked voltammetry signal was significantly and acutely decreased (by approximately 50%) after i.v. application of *para*-chlorophenylalanine (*p*-CPA). *p*-CPA is a brain-permeant 5-HT synthesis inhibitor (Koe & Weissman, 1966), which leaves CNS noradrenaline unchanged but reduces the 5-HT concentration (Ito & Schanberg, 1975). In addition, in the present experiments, α-methyl-*p*-tyrosine (AMPT) given i.v. failed to affect the evoked voltammetric signal. AMPT has been shown to be a selective noradrenaline depletor (Udenfriend *et al.*, 1965). The dose used in the present study has been shown to cause a decline after 15 min in the evoked noradrenaline release as detected by voltammetry in the ventral bed nucleus of the stria terminalis (Park *et al.*, 2011). This supports the view that the optimised voltammetric scan for 5-HT is detecting 5-HT rather than noradrenaline. In support of this, data from brainstem slices have shown that evoked EPSCs recorded from NTS neurons and caused by stimulation of the solitary tract are attenuated by blockade of 5-HT_3 receptors (Wan & Browning, 2008, Hosford *et al.*, 2013, see thesis chapter 3), while blockade of α_1-adrenceptors had no effect on the solitary tract- evoked EPSCs (Zhang & Mifflin, 2007). Thus it is unlikely that the evoked voltammetric signal contains noradrenaline.

A possible reason for the failure of blockade of the 5-HT high-affinity, low-capacity transporter (5-HTT or SERT) to increase the evoked voltammetric signal could be due to activation of 5-HT_{1A} receptors; such an interpretation has been used to explain the delay in the onset of the therapeutic effect of antidepressants (also see DISCUSSION 3.9.2). That is, although 5-HT uptake is blocked immediately the activation of 5-HT_{1A} receptors causes an initial reduction in the overall release of 5-HT in the cortex until the 5-HT_{1A} receptor desensitizes (see Artigas *et al.*, 1996). However, treatment with the archetypical 5-HT_{1A} antagonist WAY-100635 (Forster *et al.*, 1995) did not uncover a potentiating action of citalopram. Interestingly, WAY-100635 given alone caused a significant decrease in the bradycardia evoked by electrical stimulation of the vagus. This is consistent with the physiological role of 5-HT_{1A} receptors in the excitatory regulation of cardiac vagal preganglionic neurons (Bogle *et al.*, 1990, Wang & Ramage, 2001). Further, this inhibition was reversed by addition of

citalopram implying that citalopram had caused an increase in the extracellular 5−HT concentration within this pathway, although alone it had no effect on these reflexes. The site of action is probably at the level of nucleus ambiguus (Wang & Ramage, 2001), the location of cardiac vagal preganglionic neurons. This implies that the removal of extracellular 5-HT at the level of the nucleus ambiguus is different from than in the NTS.

5.12.3. Low affinity, high capacity re-uptake systems

This study has shown that blockade of the low affinity, high capacity transporters, the organic cation transporter 3 (OCT3)/plasma membrane monoamine transporter (PMAT), with decynium-22 (D-22; Schomig *et al.*, 1993, Duan & Wang, 2010) does potentiate the evoked 5-HT voltammetric signal. The failure of corticosterone (Engel & Wang, 2005), even at high doses, to effect the removal of 5-HT applied to the NTS suggests that OCT3 is not involved and the removal of 5−HT in the NTS is by PMAT (see thesis chapter 4). Interestingly, PMAT is approximately 70% more effective in transporting 5-HT compared to noradrenaline (Duan & Wang, 2010). Functionally, the increase in extracellular of 5-HT caused by D-22 was associated with significant increase in the bradycardia evoked by both vagal and cardiopulmonary stimulation, which again is consistent with the excitatory role of 5-HT in these pathways (see Ramage & Villalon, 2008). The failure to see any potentiation of reflex fall in blood pressure is presumably due to the profound hypotension caused by i.v. D-22 and explained by the α_1−adrenoceptor antagonist activity of D-22 (Russ *et al.*, 1996). It should be noted that this profound hypotensive action does not play a role in the ability of D-22 to potentiate the evoked voltammetric 5-HT signal, as a similar fall in BP caused by ganglion blocker chlorisondamine did not affect the signal. Again the ability of the blockade of PMAT, but not of the 5-HT transporter, to increase the evoked 5-HT signal is consistent with observation that the amplitude of solitary tract evoked EPSCs recorded from NTS neurons in the brainstem slice are increased in amplitude in the presence D-22 (see thesis chapter 3). In addition, the number of spontaneous EPSCs recorded after a train of solitary tract stimulation were doubled by D-22 but not citalopram (see thesis chapter 3). These combined data demonstrate

that the regulation of the increase in extracellular levels of 5-HT within NTS caused by afferent stimulation is regulated by PMAT and not the 5-HT transporter. This result differs from that reported in substantia nigra. In this brain region the increase in extracellular 5-HT evoked by stimulating the dorsal raphé, also detected by voltammetry, is under the regulation of the 5-HT transporter (Hashemi *et al.*, 2009). It should be noted that the evoked increase in extracellular 5-HT in the substantia nigra is in a similar concentration range to that recorded in the present study. A possible reason for this difference may be at the level of the NTS; 5−HT release evoked by visceral afferents involves volume transmission or paracrine transmission rather than wired transmission. The 5-HT is not being released into classical synapses but is dispersed over a larger area, an effect known as paracrine transmission (see Ramage, 2009, Fuxe *et al.*, 2013).

5.12.4. The interplay of 5-HT and glutamate release

The present data would indicate that vagal afferents cause the release of 5-HT, however glutamate is considered to be the major transmitter released by cardiovascular/visceral afferents in the NTS (see Talman, 1997, Baude *et al.*, 2009). Blockade of glutamate receptors with kynurenate (see Stone & Addae, 2002) indicated that approximately half of the signal is dependent on glutamate release from vagal afferents. This remaining signal could also be potentiated with D-22, which would be consistent with the signal identity being 5-HT. It may be argued that not all of the glutamate receptors were blocked, however increasing the dose 10-fold failed to have any further effect on the evoked 5-HT signal. However, the cardiovascular response to electrical stimulation of the vagus was reversed, a small rise in blood pressure and a tachycardia was then observed. This may simply reflect the ability of the high dose of kynurenate to penetrate further into the brainstem blocking other sites in these reflex pathways i.e. those of the ventral surface. However, it could be also be explained by the unmasking of the actions of other transmitters released during vagal stimulation, in analogy the actions of $GABA_B$ receptors in the NTS are unmasked after blockade of $GABA_A$ receptors (Wang *et al.*, 2010).

Further, blockade of Ca^{2+} channels with Cd^{2+} caused a 90% reduction of the evoked 5-HT voltammetric signal and there was nearly complete inhibition of the reflex evoked cardiovascular changes. Overall theses observation suggests that there is a glutamate-dependent and glutamate-independent source of 5−HT, which is released in response vagal afferent stimulation. This glutamate-independent release could come directly from vagal afferent terminals (see INTRODUCTION) and/or involve the release of another transmitter from the vagal afferents, such as substance P (see Helke & Seagard, 2004).

In general, the present data indicate that glutamate, presumably from vagal afferents, can cause the release of 5-HT. This is intriguing as *in vivo* (Jeggo *et al.*, 2005) and *in vitro* (Wan & Browning, 2008) experiments have shown the opposite; that 5-HT by activating 5-HT_3 receptors can cause the release of glutamate. Further, *in vitro* experiments have shown that this 5-HT-evoked glutamate release is dependent on the presence of vagal afferents (Wan & Browning, 2008), on which 5-HT_3 receptors are located. If it is assumed that at least some of the 5-HT and glutamate released in the NTS by afferent activation comes from the afferent terminals then each substance must feed forward onto other terminals to increase the release of the other substance. This would operate to consolidate the afferent input signal to the NTS. Indeed, the NTS is known to have a very low failure rate when relaying afferent input (see Andresen & Paton, 2011). This may be method of strengthening synaptic transmission.

5.12.5. Cardiovascular reflexes

It should be noted that, as activation of both chemo- and baroreceptors can increase extracellular 5-HT in the NTS, glossopharyngeal afferents are similarly structured in their ability to release 5-HT and glutamate as vagal afferents. Further, it should be noted that the fall in blood pressure caused by i.v. sodium nitroprusside failed to increase the voltammetric signal. This is not unexpected as baroreceptor afferents firing is reduced, although a decrease in the voltammetric signal may have been expected. A possible explanation why no decrease was observed might be related to the fact that the voltammetric electrodes are detecting transmitter overflow rather than

transmitter changes within a synapse. Under normal basal conditions, extracellular 5-HT concentration is tightly controlled by the high-affinity, low capacity re-uptake systems present within the NTS. This would mean that there is little efflux from release sites into the wider extracellular space, where it can be detected by voltammetry. Reducing drive by activation of the pressor reflex would do little to change the 5-HT efflux and thus the concentration measured by the electrode.

5.12.6. Conclusion

The present data demonstrate that, in the anaesthetised rat, stimulation of visceral afferents increases extracellular 5-HT levels in the NTS. This increase is due, in part, to the release of glutamate, presumably from visceral afferents. However, other possible sources such as 2nd order neurons have not been eliminated. The source of the 5-HT again has not been determined; it could be visceral afferents themselves and/or 5-HT terminals within the NTS. Published data also indicates NTS 5-HT release can itself cause the release glutamate.This 5-HT release, however surprising, is under the regulation of the high-capacity, low‑affinity transporter PMAT and not the low-capacity, high‑affinity 5-HT transporter.

This is the first demonstration that PMAT plays a physiological role in the regulation of 5-HT transmission. This might due to the fact that 5-HT is acting as a volume transmitter in the NTS. Overall these data support the view that 5-HT is an important transmitter for homeostatic regulation at the level of NTS.

5.12.7. Future experiments

It may be possible that another transmitter is released by vagal afferents, apart from glutamate, that feeds forward to activate the release of 5-HT from other sources. It has been suggested that substance P could also be released from vagal afferent terminals (see Helke & Seagard, 2004). It could, conceivably, be substance P activating NK_1 receptors on 5-HT-containing terminals within the NTS to cause the release of 5-HT

as detected by voltammetry. Potent and selective NK_1 receptor agonists, such as CP 96345 (Snider *et al.*, 1991), are available. Repeating the experiments detailed in section 5.9 but applying the NK_1 antagonist instead of D-22 may supply the necessary evidence. If substance P release is contributing to the release of 5-HT then the NK_1 antagonist would further attenuate the extracellular 5-HT concentration increase caused by vagal stimulation in the present of kynurenate.

6. 5-HT AND GLUTAMATE RELEASE IN THE NTS IN A RODENT MODEL OF HEART FAILURE: A PILOT STUDY

6.1. Introduction

In man and in animal models, the baroreflex and the cardiopulmonary reflex are reportedly impaired in early heart failure (Sved *et al.*, 2003, Modesti *et al.*, 2004). Further, inappropriate alterations in these reflexes have been considered to underlie the majority of cases of hypertension (Guyenet, 2006, Flanagan *et al.*, 2008). Maladaptive changes within systems governing cardiovascular reflexes can exacerbate heart failure and contribute to the progression of the disease (Kaye & Esler, 2005). The processing of these reflexes requires the release of various neurotransmitters within the NTS and an understanding of how different chemical transmitters are used here could be crucial to gaining insights into the failure of homeostatic mechanisms in heart failure.

Now with an understanding of some of the basic physiology of 5-HT release in the NTS under normal physiological conditions it was hypothesised that during abnormal cardiovascular function 5-HT release would be modified. A rat model of post-myocardial infarction-induced heart failure is available and has been used to investigate differences in signalling within the brainstem during heart failure (Marina *et al.*, 2013). In the present study experiments were conducted to determine the effect of infarct-induced heart failure on the properties of 5-HT and glutamate released during activation of cardiovascular reflexes.

6.2. Specific Aims

- Validate the model of post-myocardial infarction-induced heart failure by assessing the pathophysiology of the heart in both sham and coronary artery-ligated rats.

- Determine if the properties of 5-HT release in the NTS caused by a range of frequencies of electrical vagal stimulation is modified in heart failure.

- Investigate the differences in 5-HT release in the NTS during activation of the cardiopulmonary reflex in heart failure.

- Investigate the changes in glutamate release caused by activation of the depressor reflex in heart failure.

6.3. Supplementary methods

Rat model of myocardial infarction-induced heart failure

Myocardial infarct was induced by ligating the coronary artery according to the technique described in detail elsewhere (Pfeffer *et al.*, 1979, 1991, Francis *et al.*, 2001). Briefly, rats 180-210g (n = 18) were anaesthetised with a mixture of ketamine (60 mg kg^{-1}; i.m.) and medetomidine (250 μg kg^{-1}, i.m.), an orotracheal cannula was put in place and the rat was artificially ventilated with O_2-enriched room air. The heart was exteriorised *via* a left thoracotomy and the pericardium removed. The left anterior descending coronary artery was ligated on the border of the pulmonary outflow tract and the left atrium using a 5–0 Merisilk suture. The perfusion of the left ventricle was monitored visually by colour change to confirm successful occlusion of the coronary artery. Sham operated rats underwent exactly the same procedure except that the suture was passed around the coronary artery but not tied. The heart was returned to the thoracic cavity and the chest incision closed. Penicillin-streptomycin-containing powder was applied to the incision site and anaesthesia was reversed with atipamezole (1 mg kg^{-1}). 3 of the 18 animals died within 48 hours of surgery due to sudden cardiac death, as is common with this procedure. After at least 14 days post surgery the experimental animals were set up for measurement of 5-HT as described in METHODS 2.1.

Histology

Upon completion of the experiment rats were sacrificed with an overdose of pentobarbitone (30 mg kg^{-1}). The heart was removed and weighed immediately. The left ventricle was then removed with fine dissection scissors and wrapped in a plastic paraffin film to prevent dehydration. The tissue was then frozen at 20 °C for 20 min and then sectioned from the apex to the base into four transverse slices of approximately 1.5 mm. The sections were then immersed in a solution of 1% (w/v) triphenyl tetrazolium chloride (TTC) in 0.01M phosphate buffered saline. The solution was incubated at approximately 37 °C for 15-20min. The sections were then transferred to a 10% formalin solution overnight before being photographed. The area of the ventricle lumen and muscle was determined using computerised planimetry.

Measurement of extracellular glutamate

Experimental animals were set up as described in METHODS 2.1 up to the point of electrode implantation. For the measurement of extracellular glutamate concentration changes enzyme-encapsulated electrodes were used as described elsewhere (Gourine *et al.*, 2008, Tian *et al.*, 2009). The glutamate sensing electrodes, the 'active electrode', contains glutamate oxidase encapsulated in a matrix coated on a platinum wire (diameter 50μm, length 1mm). Glutamate oxidase converts glutamate to α-ketoglutarate along with NH_3 and H_2O_2. The platinum wire is held at a constant voltage of +500mV, sufficient to oxidise the H_2O_2. Increases in glutamate concentration feed-forward to increases in H_2O_2 and, in turn, increases current flux through the platinum electrode. The electrode is protected from interfering electroactive species, such as 5-HT by a permselective membrane. Additionally, a dual recording configuration was used to control for unknown oxidisable interferents. In addition to the 'active' glutamate electrode, a 'null' electrode was implanted in an equivalent position in the contralateral NTS. This electrode was identical but did not contain glutamate oxidase. Current changes at this electrode were subtracted from the current changes from the active electrode.

Specificity of glutamate electrodes

The glutamate biosensor used in this study has been extensively validated for specificity for glutamate (Tian *et al.*, 2009). The authors reported a relative response of a range of common interfering substances, including 5-HT, of less than 10% when compared to glutamate. They also reported that the sensor is unresponsive to changes in pH in the range expected to be encountered within the brain during intense neuronal activity. Further, the sensor retained sensitivity in low oxygen tension environment.

6.4. Results: Pathology

Morphology and metabolic activity

14 days after coronary artery ligation gross morphology of the left ventricle shows an enlarged ventricle with thinning of the external ventricle wall (Figure 6.1). When stained with redox indicator TTC the external left ventricle wall near the site of infarct remained white and that taken up by the remaining tissue was reduced to the red 1,3,5-triphenylformazan (TFP). In comparison the left ventricle section from the sham-operated rat shows no thinning of the left ventricle wall and deep red staining from TFP accumulation in the tissue. Note that the tissue from the sham operated heart stained a much deeper red than that from the ligated heart.

Heart weight and ventricular ratio

Hearts removed from sham-operated rats (n=6) had a wet weight of 3.3 ± 0.1g kg^{-1} of body weight. This was significantly increased (P<0.05) in rats with ligated hearts (n=8), which had a wet weight of 3.8 ± 0.2g kg^{-1} of body weight (Figure 6.2A). There was no significant difference in the wet weight of the ventricle in sham operated (2.1 ± 0.04g kg^{-1}) and ventricles of ligated hearts (2.3 ± 0.1g kg^{-1}).

The ratio of left ventricular lumen cross sectional area to ventricular wall area was 0.05 ± 0.003 in hearts removed from sham-operated rats. This was significantly greater (P<0.001) in hearts removed from ligated hearts, which had a ratio of 0.3 ± 0.03 (Figure 6.2B).

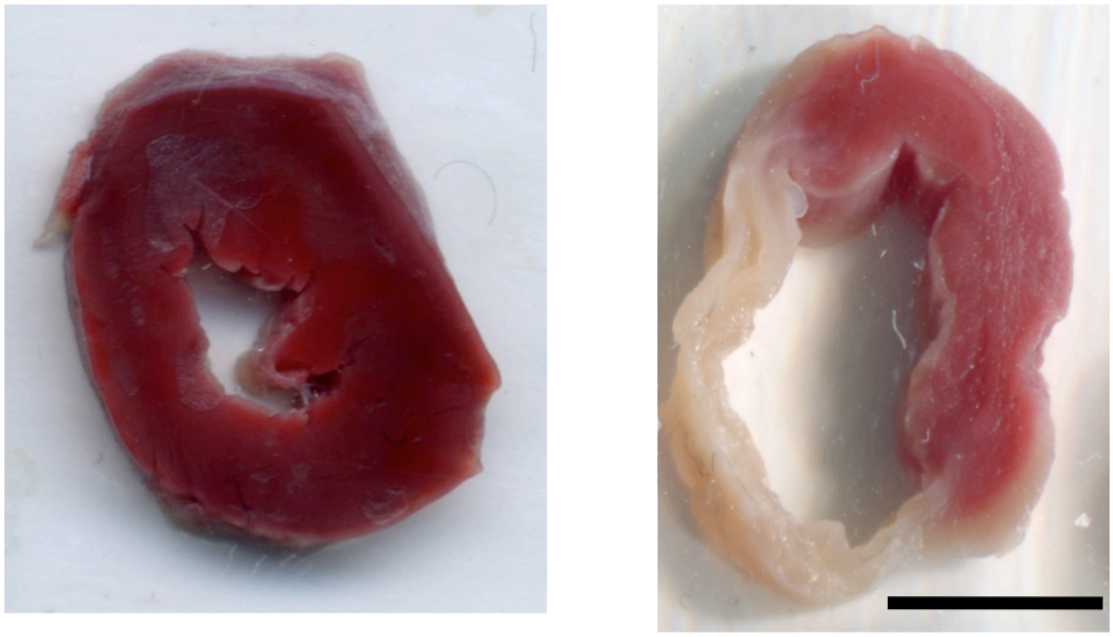


Figure 6.1 Heart Failure pathology: Histology

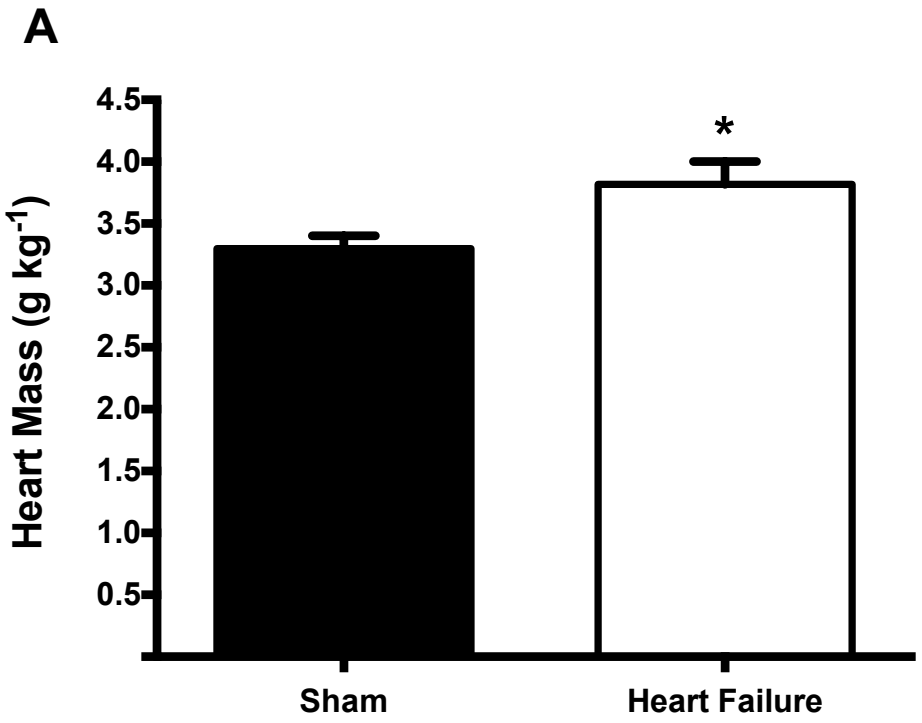
Photographs of two left ventricles stained with TTC taken from (*left*) a sham operated rat heart and (*right*) a rat heart with coronary vessel ligation. Both were sacrificed after 14 days. Cross sections are approximately 1.5mm thick and were taken the centre of the ventricle. Scale bar represent 5mm.

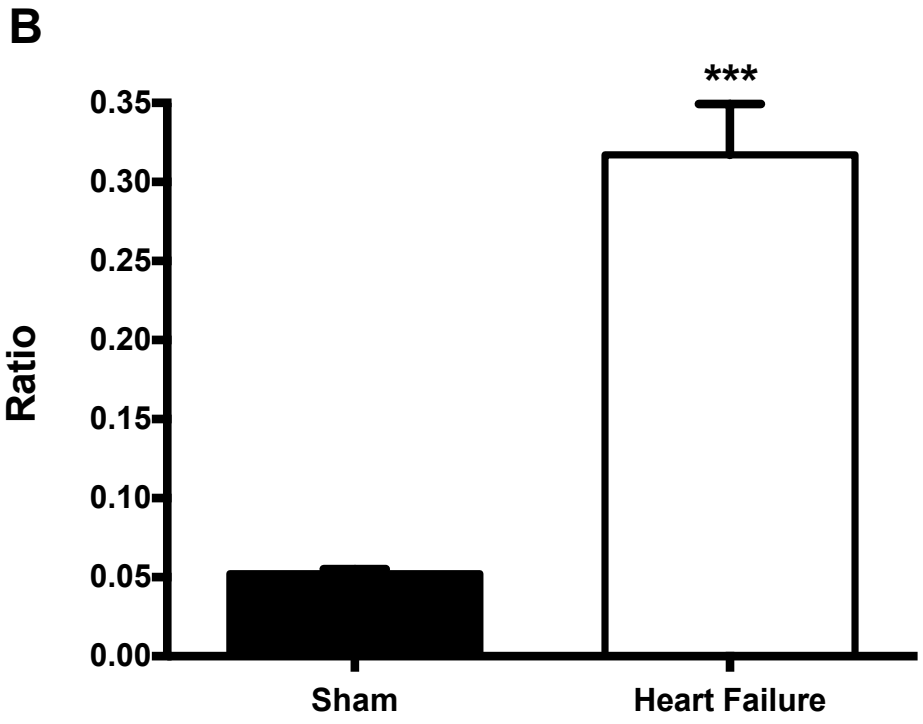
Figure 6.2 Heart Failure pathology: Histograms

Rat model of post-myocardial infarction-induced heart failure:

A) Histogram comparing wet heart mass of sham-operated rats (Sham; n=6) and coronary artery ligated rats (Heart Failure; n=8) sacrificed 14 days post-procedure. *P<0.05, Compared to control with Student's paired t-test.

B) Histogram comparing the ratio of left ventricular lumen cross sectional area to ventricular wall area in sham operated (Sham; n=6) and coronary artery ligated rats (Heart failure; n=8) sacrificed 14 days post-procedure. ***P<0.001, Compared to control with Student's paired t-test.


A
Heart Mass (g kg-1)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
*
Sham
Heart Failure


B
Ratio
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
Sham
Heart Failure

6.5. Baseline haemodynamic values

Heart rate measured over 5 min after induction of anaesthesia in animals that had undergone coronary ligation (n=8) was 356 ± 11bmp. This was not significantly different from that measured in sham-operated rats (n=6), which had a mean heart rate of 372 ± 14bmp.

Mean arterial blood pressure measured over the same 5min period after induction of anaesthesia in animals that had undergone coronary ligation was 101 ± 4mmHg. This was found to be significantly different ($P<0.05$, Compared with Student's paired *t*-test) from sham-operated animals, which had a mean arterial blood pressure of 121 ± 9mmHg.

6.6. Electrical stimulation of the vagus in heart failure

Extracellular 5-HT concentration in the NTS

When the vagus nerve was stimulated at 10x threshold using frequencies of 10, 20 and 50Hz, extracellular increases in 5-HT were 18.4 ± 3 nM, 50.5 ± 8.1nM and 82.4 ± 21.4nM, respectively in animals that had undergone coronary ligation (n=3). In animals that had undergone the sham procedure stimulating at 10x threshold using frequencies of 10, 20 and 50Hz caused extracellular increases in 5-HT of 25 ± 11.5 nM, 52.5 ± 7.0nM and 53.8 ± 12.0nM, respectively (Figure 6.3. No significant differences were found between the two groups at any stimulation frequency when compared with Student's *t*-test.

Cardiovascular

Vagus nerve stimulation at 10x threshold using frequencies of 10, 20 and 50Hz caused bradycardia of 17 ± 8 bpm, 22 ± 8 bpm and 43 ± 11 bpm, respectively in animals that had undergone coronary ligation (n=3). This was not significantly different from animals that had undergone the sham procedure, in which the same simulation parameters of 10, 20 and 50Hz now caused bradycardia of 29 ± 11 bpm, 31 ± 12 bpm and 32 ± 3 bpm.

When the vagus nerve was stimulated at 10x threshold using frequencies of 10, 20 and 50Hz hypotension of 20 ± 4 mmHg, 31 ± 8 mmHg and 34 ± 12 mmHg, respectively was produced in animals that had undergone coronary ligation (n=3). This was not significantly different from animals that had undergone the sham procedure, in which the same simulation parameters of 10, 20 and 50Hz now caused hypotension of 18 ± 4 mmHg, 25 ± 4 mmHg and 33 ± 3 mmHg.

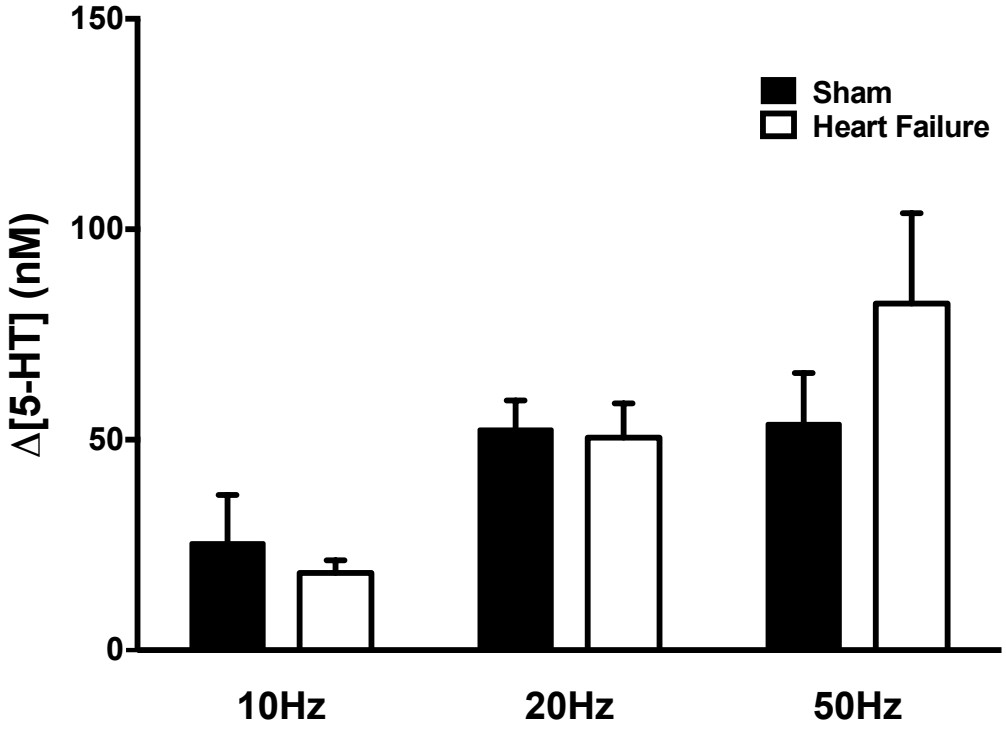


Figure 6.3 Heart Failure Model: Histograms

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the different frequencies of electrical stimulation of the central end of the left vagus (1ms pulse-width, 10s duration) at 3 min intervals at different frequencies and 10x threshold (threshold range was between 70-110 μA) on changes (Δ) in the 5-HT concentration in the NTS as detected by fast cyclic voltammetry. Each column is the mean (±s.e.m) data from 3 rats in each experimental group.

6.7. Results: Cardiovascular reflexes

Cardiopulmonary reflex

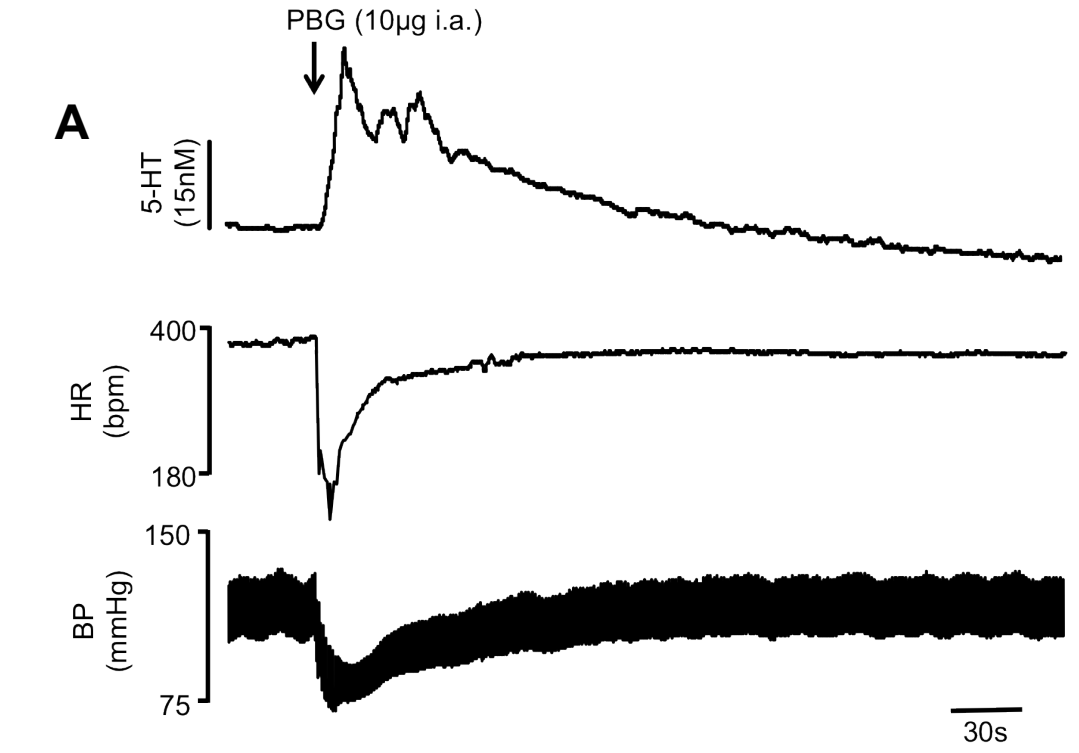
In animals that had undergone coronary artery ligation, activation of the cardiopulmonary reflex by intra atrial (i.a.) injection of the 5-HT_3 receptor agonist phenylbiguanide (10µg per animal) caused an increase in extracellular 5-HT of 29 ± 6nM. This was significantly different ($P< 0.05$) from sham-operated animals, in which by i.a. injection of phenylbiguanide caused an increased in extracellular 5-HT of 10 ± 0.4nM (Figure 6.5).

The decrease in blood pressure and the decrease in heart rate caused by i.a. injection of phenylbiguanide were 34 ± 5mmHg and -148 ± 39bpm in animals that had undergone coronary artery ligation. In sham-operated animals these changes were 58 ± 6mmHg and -191 ± 12bpm. The hypotension was found to be significantly decreased in rats that had undergone coronary ligation ($P< 0.05$).

All data represent n=4 taken from two animals in each experimental group (2 ligated, 2 sham). Representative experimental traces are shown in figure 6.4.

Figure 6.4 5-HT release in heart failure: Cardiopulmonary reflex traces

An anaesthetised artificially ventilated, neuromuscular-blocked male rats A) having undergone coronary artery ligation and B) having undergone a sham procedure showing an increase in the 5-HT concentration in the NTS, a decrease in arterial blood pressure (BP) and decrease in heart rate (HR) caused by an i.a. injection of phenylbiguanide (PBG; 10μg).


PBG (10µg i.a.)
A
5-HT
(15nM)
400
HR
(bpm)
180
150
BP
(mmHg)
75
30s

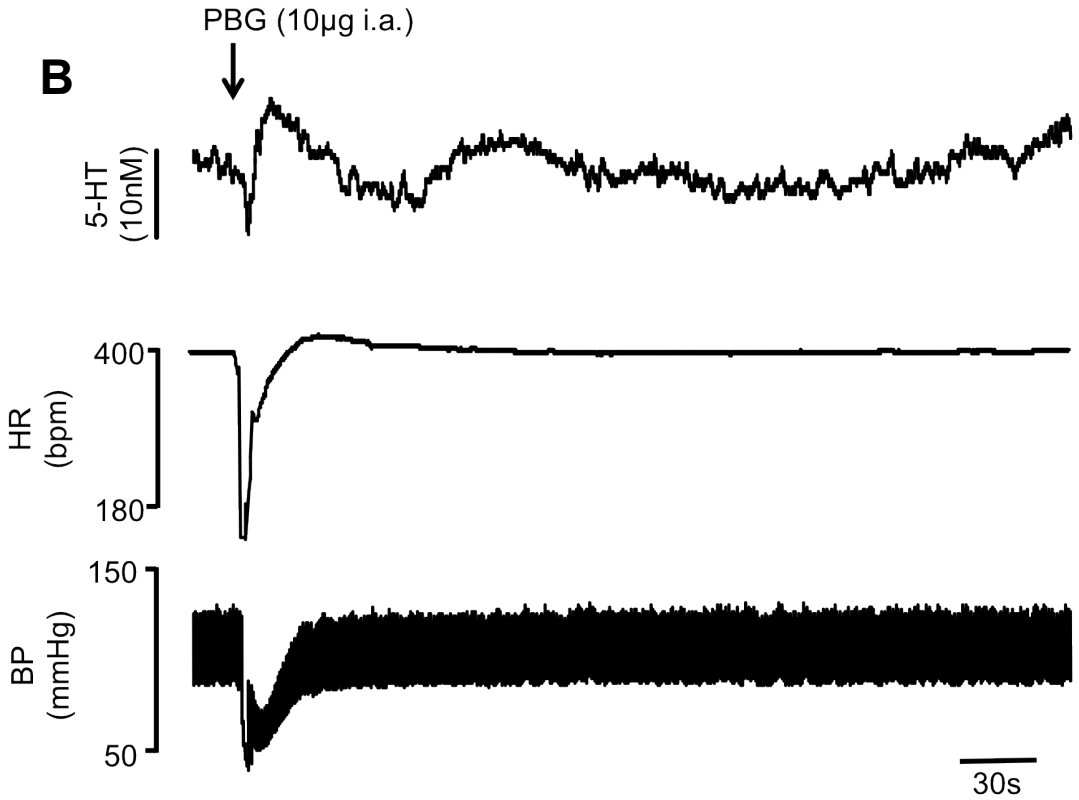
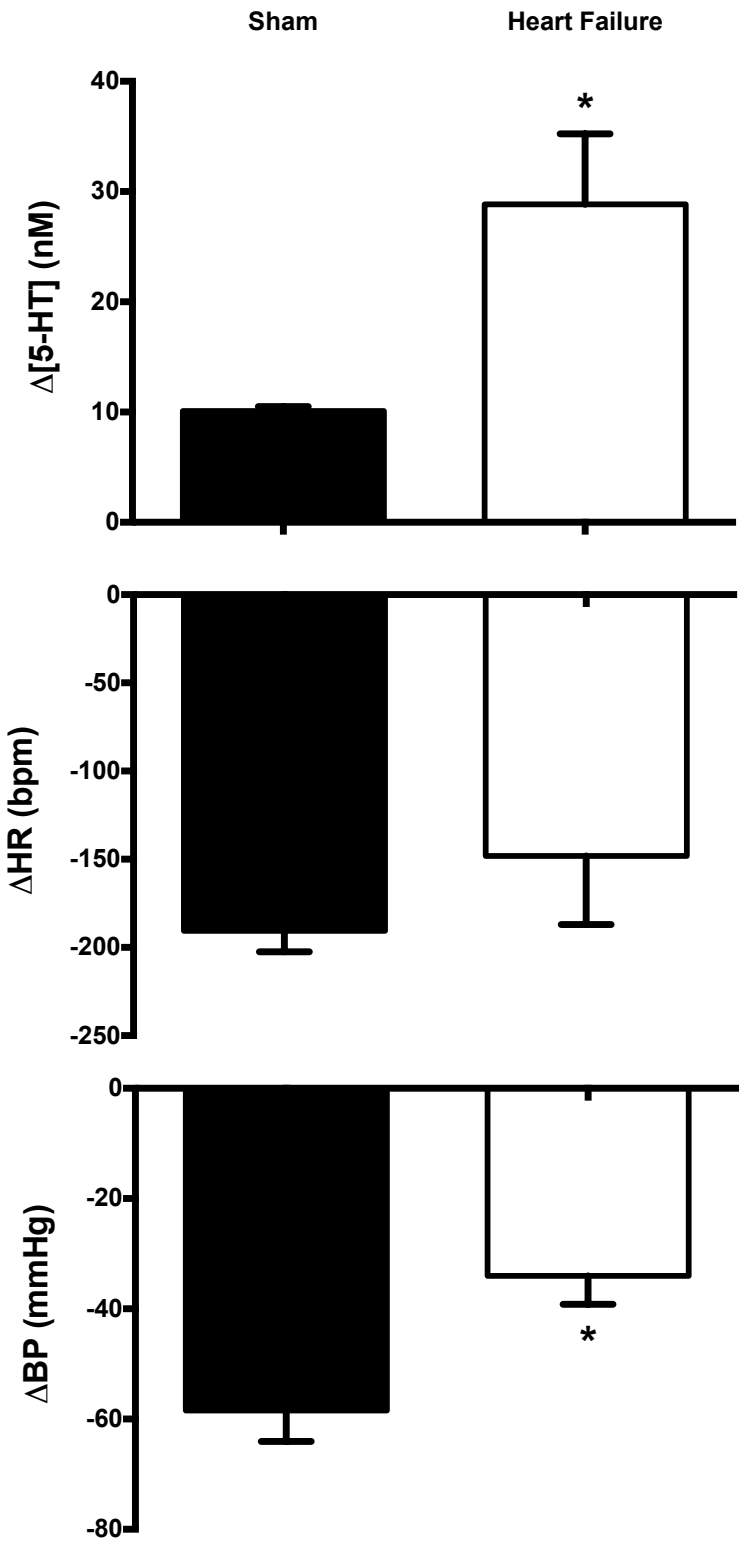

PBG (10µg i.a.)
B
5-HT
(10nM)
400
HR
(bpm)
180
150
BP
(mmHg)
50
30s

Figure 6.5 Heart Failure Model: Cardiopulmonary reflex histograms

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects on mean increases in extracellular 5-HT concentration ([5-HT]) in the NTS and the decreases in mean arterial blood pressure (BP) and heart rate (HR) caused by i.a. injection of the 5-HT_3 agonist phenylbiguanide (10μg per animal) in animals that had undergone coronary artery ligation (Heart Failure; n=4 in 2 animals) or sham procedure (Sham; n=4 in 2 animals). The bars show the s.e.m. These changes were compared with unpaired Student's *t*-test. * $P<0.05$.


Sham
Heart Failure
Δ[5-HT] (nM)
40
30
20
10
0
*
ΔHR (bpm)
0
-50
-100
-150
-200
-250
ΔBP (mmHg)
0
-20
-40
-60
-80
*

6.8. Results: Glutamate release in heart failure

In animals that had undergone coronary artery ligation (n=3) activation of the depressor reflex by i.v. injection of the α_1-adrenoceptor agonist phenylephrine (3μg per animal) caused an increase in extracellular glutamate 1.6 ± 0.2μM that was significantly different ($P< 0.05$) when compared to sham-operated animals (n=3), in which i.v. injection of phenylephrine caused an increased in extracellular glutamate by 0.6 ± 0.2μM (Figure 6.7).

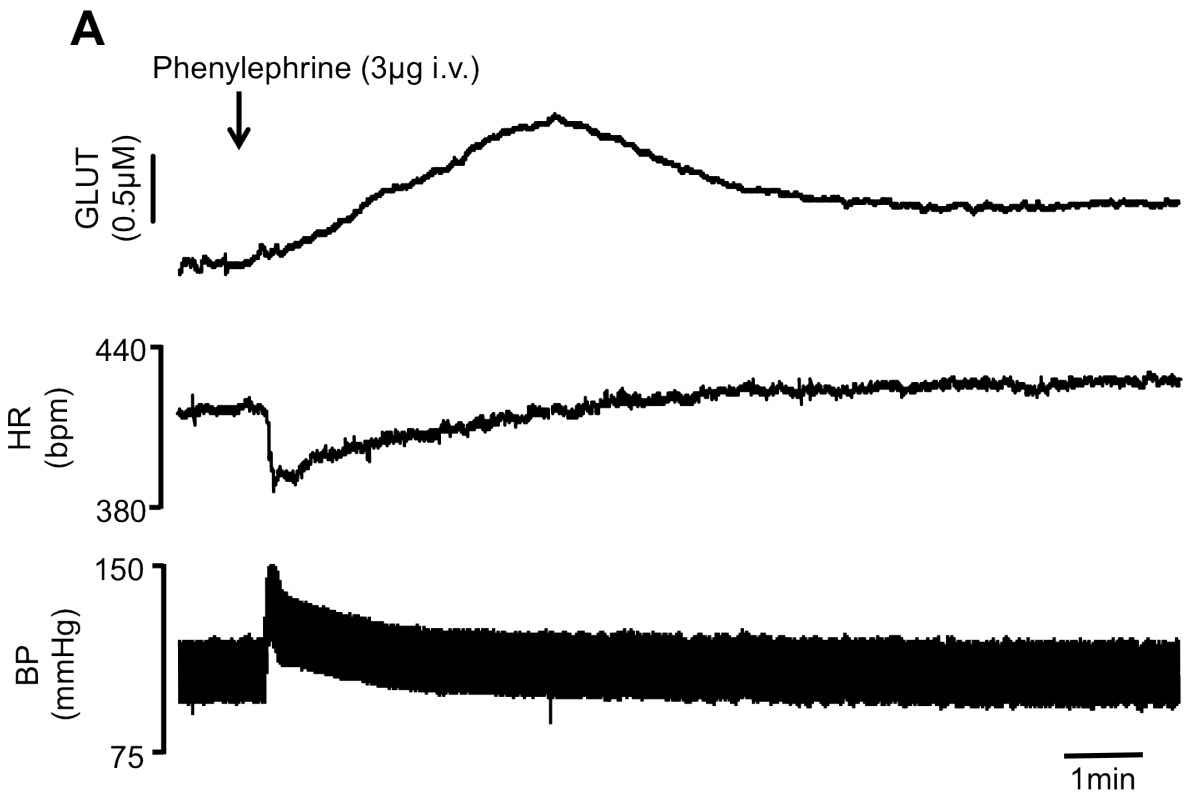
The increase in blood pressure and the decrease in heart rate caused by i.v. injection of phenylephrine were 46 ± 2mmHg and -25 ± 4bpm in animals that had undergone coronary artery ligation. In sham-operated animals these changes were 49 ± 8mmHg and -43 ± 12bpm. There were no significant differences found between the two experimental groups.

Representative experimental traces are shown in figure 6.6.

Activation of the pressor reflex by i.v. injection of nitric oxide donor vasodilator sodium nitroprusside (SNP; 50μg per animal; n=2) was found to have no effect on extracellular glutamate concentration in the NTS in animals that had undergone coronary artery ligation or sham procedure (Figure 6.8).

Figure 6.6 Glutamate release in heart failure: Depressor reflex trace

An anaesthetised artificially ventilated, neuromuscular-blocked male rat having undergone coronary vessel ligation showing an increase in the glutamate concentration in the NTS, an increase in arterial blood pressure (BP) and decrease in heart rate (HR) caused by an i.v. injection of phenylephrine (3μg).


A
Phenylephrine (3µg i.v.)
GLUT
(0.5µM)
HR
(bpm)
440
380
BP
(mmHg)
150
75
1min

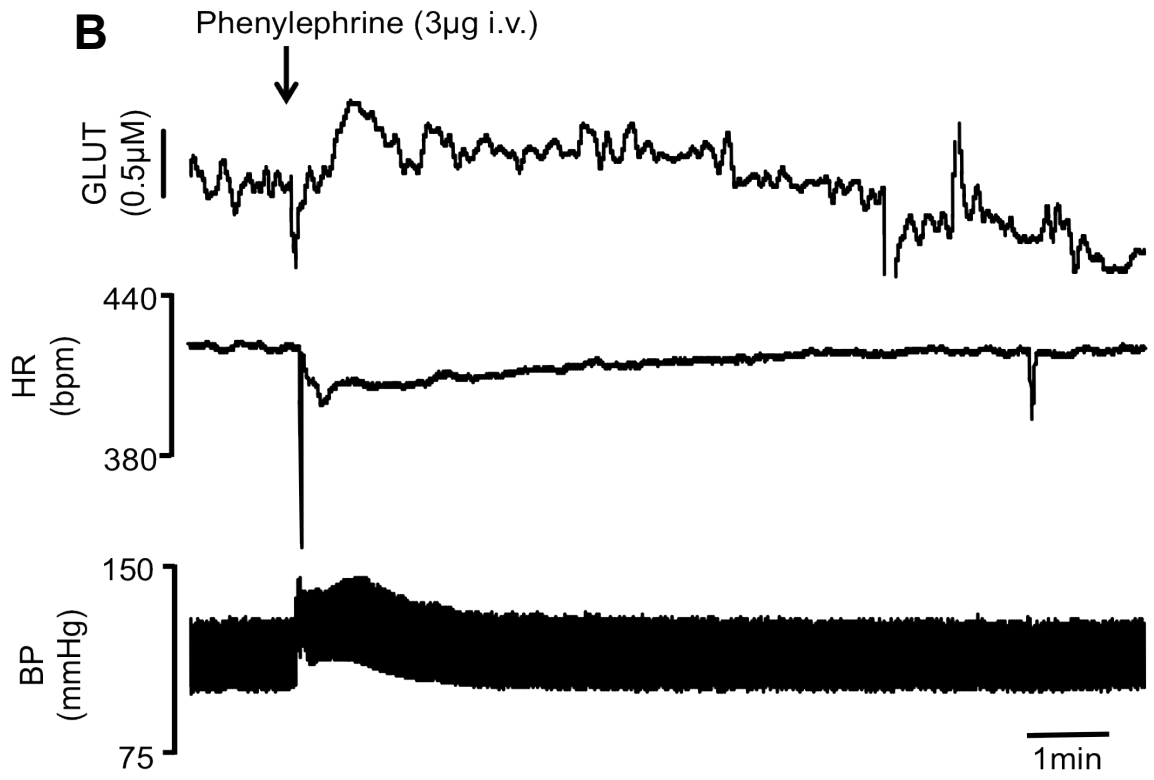
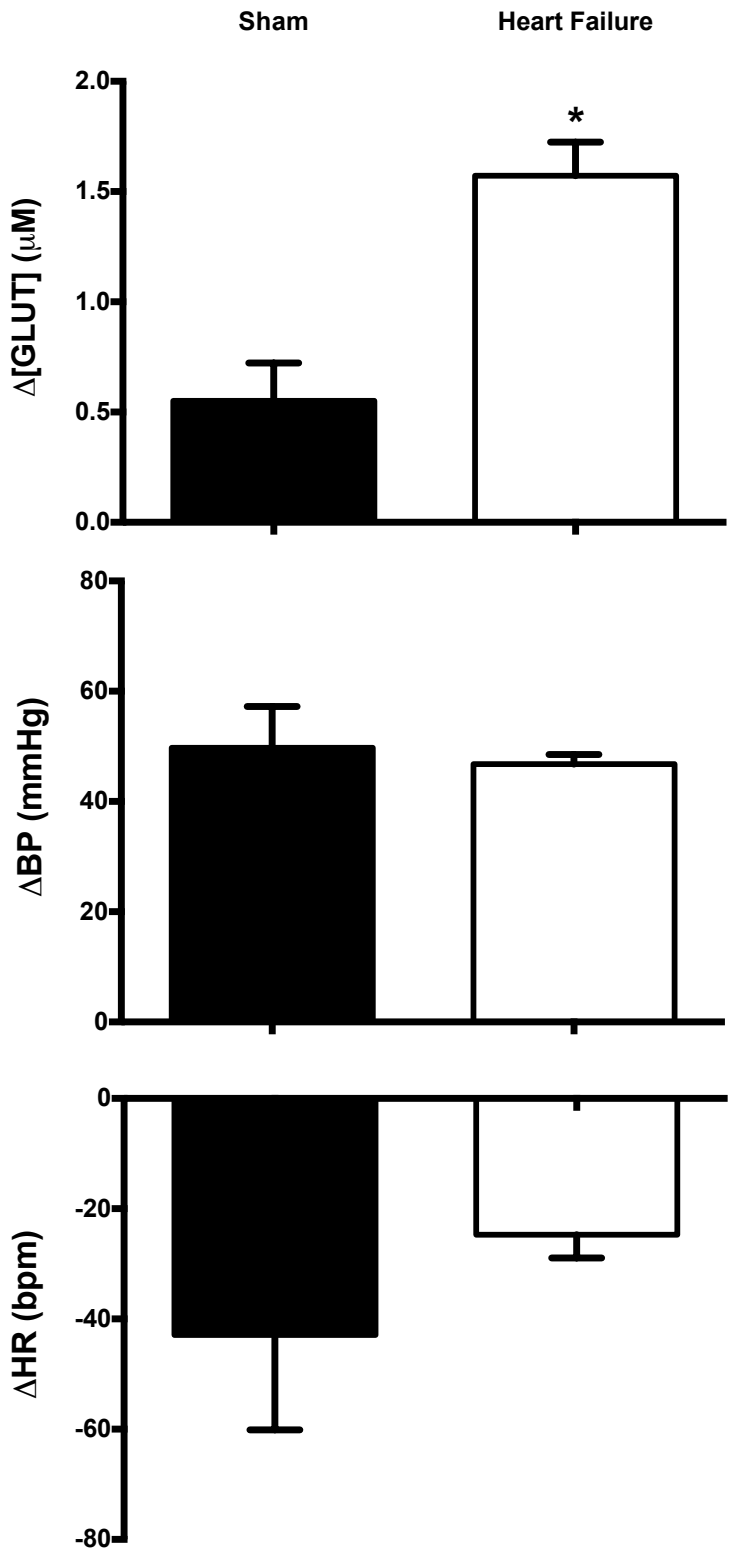

B
Phenylephrine (3µg i.v.)
GLUT
(0.5µM)
HR
(bpm)
440
380
BP
(mmHg)
150
75
1min

Figure 6.7 Glutamate release in heart failure: Histograms

Anaesthetised artificially ventilated, neuromuscular-blocked male rats: histograms comparing the effects on mean increases in extracellular glutamate concentration ([GLUT]) in the NTS and the increases in mean arterial blood pressure (BP) and decreases in heart rate (HR) caused by i.v. injection of the α_1-adrenoceptor agonist phenylephrine (3μg per animal) in animals that had undergone A) coronary artery ligation (Heart Failure, n=3) or B) sham procedure (Sham, n=3).The bars show the s.e.m. These changes were compared using unpaired Student's *t*-test. * $P<0.05$.


Sham
Heart Failure
2.0
1.5
1.0
0.5
0.0
Δ[GLUT] (μM)
*
80
60
40
20
0
ΔBP (mmHg)
0
-20
-40
-60
-80
ΔHR (bpm)

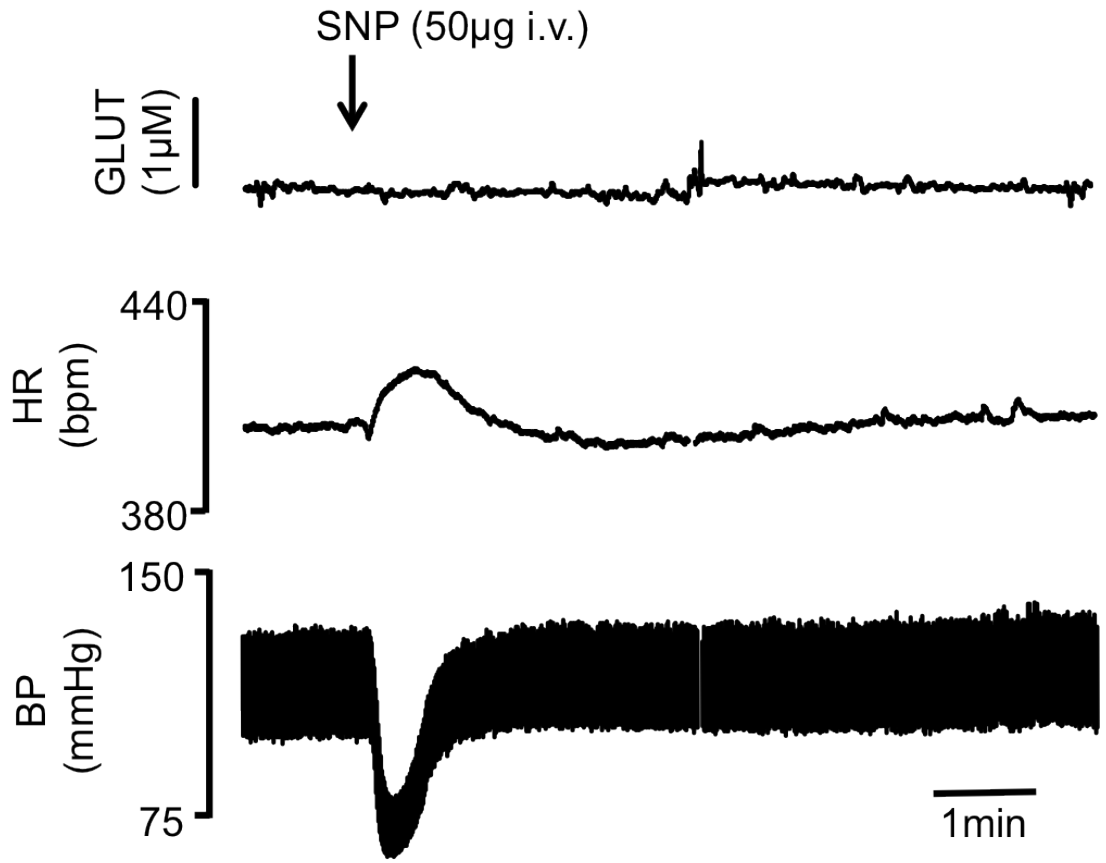


Figure 6.8 Glutamate release in heart failure: Pressor trace

An anaesthetised artificially ventilated, neuromuscular-blocked male rat showing no change in the glutamate concentration in the NTS, a decrease in arterial blood pressure (BP) and increase in heart rate (HR) caused by an i.v. injection of sodium nitroprusside (SNP; 50µg).

6.9. Discussion

6.9.1. Main findings

Activation of the cardiopulmonary reflex was found to cause an approximately 3-fold increase in extracellular 5-HT in animals with heart failure compared with those that had undergone the sham procedure. Equally, the increase in extracellular glutamate concentration evoked by activation of the depressor reflex was found to be higher in animals with heart failure than in those that had undergone the sham procedure. Interestingly, when the increase in extracellular 5-HT increased is evoked by electrical stimulation there is no difference between the two experimental groups. Lastly, activation of the pressor reflex failed to modify extracellular glutamate concentrations in both experimental groups.

6.9.2. Conclusion

The present study suggests that in heart failure caused by myocardial infarction, glutamate and 5-HT transmission are augmented within the NTS when the depressor reflex and the cardiopulmonary are activated, respectively. This conclusion is supported by the data obtained from direct, real-time electrochemical measurement of the two transmitters within the NTS during reflex activation. These data suggest that compensatory mechanisms are activated centrally to modulate cardiovascular reflexes. Whether these changes are maladaptive and they have an impact on the progression of the pathology is yet to be determined.

6.9.3. Future experiments

As this was only a preliminary set of experiments using this rodent model of heart failure, a full investigation of changes seen in the 5-HT and glutamate released during reflex activation is necessary. The changes in glutamatergic transmission would be of particular interest.

7. GENERAL DISCUSSION

7.1. Conclusions

The present experiments have investigated the neuropharmacology of the release and re-uptake of 5-HT both *in vitro* and *in vivo* with respect to the afferent processing within the NTS. Efforts have been made to verify the pharmacological selectivity and effectiveness of the compounds used to manipulate the monoamine re-uptake mechanisms in the NTS. Additionally, experiments have begun to assess the changes in the regulation of transmitter release as a result of chronic heart failure that may occur in the NTS. From the data it is possible to conclude the following points:

1) 5-HT is released within the NTS upon stimulation of vagal afferents. At least half of the detected 5-HT is not due to glutamate release and may be directly released by vagal afferent terminals within the NTS. The remaining 5-HT is released from other sources that require glutamate to be released from vagal afferents. This indicates that 5-HT terminals located within the NTS, arising from medullary raphé, must be also activated by vagal input to release this 5-HT. Is should be noted that 5-HT has also been demonstrated to release glutamate the NTS. These combined data imply that 5-HT and glutamate must feed forward onto other terminals to increase the release of each other. This form of synaptic consolidation may serve to improve reliability of transmission within the NTS.

2) A low-affinity, high-capacity monoamine uptake transporter, probably PMAT rather than OCT3, and not the high-affinity, low-capacity 5-HT transporter is involved in the regulation of the rise in extracellular concentration of 5-HT in the NTS in response vagal afferent stimulation. This is evident both in the brainstem slice and from the anaesthetized rat preparation. This could be indicative of 5-HT acting as a volume transmitter within the NTS.

3) In the NTS slice, augmenting extracellular 5-HT concentration with re-uptake inhibitors unmask a presynaptic 5-HT_{1A} receptor that is inhibitory to glutamate release. This is effect persists despite an abundance of presynaptic 5-HT_3 receptors,

which are excitatory. In this arrangement 5-HT_{1A} receptors are protected from activation by re-uptake systems. This may be because the 5-HT_{1A} receptor is located remote from the sites where 5-HT is released. This is further evidence that 5-HT acts as a volume transmitter within the NTS.

4) Re-uptake inhibitors are often used to clarify the identity of an unknown electrochemical signal. However, little data exists regarding the acute effects, dosing and selectivity of these drug when used *in vivo*. Direct assessment of a range of re-uptake inhibitors has shown that citalopram and desipramine are selective for 5-HT and noradrenaline, respectively, at doses of 1mg kg^{-1} when administered i.v. *in vivo*.

5) Heart failure causes an augmentation of glutamate and 5-HT release within the NTS. These neurogenic changes could represent a maladaptive compensation and may contribute to disease progression.

7.2. Limitations

Anaesthesia

In vivo experimental design must always balance the level of control afforded by the use of anaesthesia and neuromuscular blockade with the desire to maintain as close to normal physiology as possible. The experiments presented in chapters 4, 5 and 6 all use the anaesthetised rat preparation. As a result confounding factors such as animal movement, changes in ventilation and blood gases were controlled or removed.

However, while anaesthesia allows for more invasive and technically advanced experiments to be carried out on the whole animal, it must be recognised that anaesthetic drugs are themselves a confounding factor. Cardiovascular reflexes function to maintain the physiology of awake animals. As anaesthetics all have depressant actions on neural circuits controlling these reflexes, so cannot be expected to behave as they would in the absence of these drugs. Nevertheless, care has been taken to minimise the impact by the selection of an anaesthetic agent, α-chloralose,

widely used in this type of research due to its low depressant action on cardiovascular, respiratory, and reflex functions (Nakao *et al.*, 2001). However, it is still known to alter the physiology of some aspects of the cardiovascular system. For example baroreflex and chemoreflex are blunted by chloralose anaesthesia when compared to awake animals (Covert *et al.*, 1988, Kellett, 2005). It should also be noted that only a few studies have investigated cardiovascular reflexes in awake animals due to technical difficulty and limitations. This makes using anaesthetised preparations more advantageous as direct comparisons with the established literature can be made.

It may be possible in future studies to perform voltammetry in the awake rat. This has already been achieved for dopamine (Rebec *et al.*, 1997) and a technique developed by Michelini and Bonagamba (1988) would allow implantation of microelectrodes into the NTS of awake, freely moving and unrestrained rat.

Brainstem slice preparation

The brainstem slice preparation used in this thesis avoids the need for anaesthesia completely. However, there are other significant limitations of the brainstem slice preparation when compared to studying NTS function in the intact brain. Firstly, the ability to relate end-organ functional changes (heart rate, blood pressure) to neurophysiological changes seen in the brain is impossible. Secondly, the brain requires networks of neurons to function and preparation of the slice will remove many inputs to the NTS. Therefore, it is important to interpret results accordingly and use the data obtained in slice preparations to design *in vivo* studies to support any conclusions.

7.3. Future studies

Determining the contribution of glia to the spontaneously released glutamate in the NTS would be the focus of one future experiment. The present study used a metabolic inhibitor in an attempt to dissect out functional aspects of the glutamate release from

glia. Although this is an important first step in elucidating the role of glia in the NTS, a more specific and versatile method of manipulating glial function is necessary to explore their role fully. To this end, it is possible to specifically target glia with an adeno-associated viral vector (AAV) using a glial-specific promoter. Glial fibrillary acidic protein (GFAP) is a cytoskeletal component abundantly expressed in astrocytes but, importantly, not expressed by neurons (Brenner *et al.*, 1994). Vectors with this promoter have been used recently to express channel rhodopsin (ChR) in astrocytes. ChR is a light sensitive nonspecific cation channel and, when activated, facilitate a low flux of calcium across the cell membrane, which can then trigger calcium waves and subsequent astroglial signalling (see Gradinaru *et al.*, 2007). Such an experimental approach has been used to great effect in the brainstem to demonstrate respiratory responses were triggered when astrocytes were specifically stimulated (Gourine *et al.*, 2010).

Additionally, it is possible to introduce an inhibitory G-protein-coupled receptor (G_i) that is activated by an inert ligand, known as DREADDs (designer receptors exclusively activated by designer drug), using the same vial vector with a GFAP promoter (see Wess *et al.*, 2013). Expression of G_i coupled DREADD has been used previously to silence excitable cells *in vivo* and *in vitro* by inhibiting cAMP production (Ray *et al.*, 2011). Using this technique it may be possible to temporarily inhibit the release of gliotransmitters.

Using these two experimental approaches, first *in vitro*, it would be possible to determine the contribution of the glia to the spontaneously released glutamate as recorded in the NTS-containing brainstem slice preparation. Secondly, by silencing the astrocytes while applying 5-HT_3 agonists it would be possible to determine if astrocytes are activated and contribute to the increase in mEPSCs caused by 5-HT_3 activation.

This experimental approach can also be used *in vivo* to selectively activate 5-HT-containing cells within the NTS. A specific promoter can be used to limit the expression of rhodopsin to cells that express 5-HT. More selective activation/ inhibition of these cells would allow the physiological role of 5-HT within the NTS to be elucidated.

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